IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Arlene RAMSINGH *et al.*

Serial No.: 09/879,572

Filing Date: June 12, 2001

For: COXSACKIEVIRUS B4 EXPRESSION VECTORS AND USES THEREOF

Examiner: Stacy Chen

Group Art Unit: 1648

Atty Dkt: 29025.0001

Customer No.

26694

PATENT TRADEMARK OFFICE

DECLARATION OF STEVEN TRACY, Ph.D., PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, the undersigned, declare as follows:

1. I am an expert in the field of viral biology and molecular virology and have worked with coxsackieviruses and other Picornaviruses for 25 years. I am an author/co-author of about 85 peer-reviewed publications, review articles and book chapters in this field. My *Curriculum Vitae* is attached. I am currently a Professor of Pathology & Microbiology at the University of Nebraska Medical Center. I am very familiar with the research publications of Dr. Arlene Ramsingh, co-inventor of the above-identified patent application. We are colleagues in the same field.

2. I have reviewed the relevant sections of the outstanding Office Action and the patent application, including the rejected claims. My comments below are addressed to several points raised by the Examiner, particularly in relation to her citation of the reference Caggana, M, Chan, P, and Ramsingh, A (1993) *J. Virol.* 67:4797-4803 (referred to below as "Caggana") as anticipating claims 1, 3, 4, 18, and 20-26.

3. I wish to preface my specific remarks with the following general statements. Simply put, virus populations, especially RNA virus populations in which no editing function exists in the RNA-dependent RNA polymerase, are constantly changing. The extent to which change occurs is a function of how the virus population at present adapts to the current environment. Merely inoculating cells with a dose of an RNA virus results in a new population of virus in which changes in the RNA can be found which were not present prior to the passage. Virus populations are not cast in concrete, but are plastic. Such differences do not necessarily make non-identical viruses "heterologous" to one another. It is important to keep this in mind when trying to "equate" notions of homology and heterology in viruses, especially RNA viruses, with the similar concepts applied to DNA whether from *E. coli*, yeast or mammalian cells. It is hoped that my remarks will help clarify these issues.

4. As regards viruses of the CB4 serotype, JVB can be considered a strain of the CB4 *serotype*; CB4-P and CB4-V can be considered either variants of that strain, variants of one another or different strains. That distinction is not really helpful. Because CB4-V was derived from CB4-P by mouse passage, the CB4-V strain or variant is clearly very closely related "structurally" to the CB4-P strain or variant, sharing near-perfect identity. They may be considered "homologues" of one another; they certainly are not heterologous. So even though they lack 100% sequence identity, CB4-V and CB4-P cannot be viewed as heterologous to one another in terms of viral biology. It is well known in the picornavirus field that genetic variations *within* a particular group can have diverse biological or "clinical" phenotypes. For example, at certain doses, avirulent or non-pathogenic genetic variants of the JVB strain of CB4, like CB4-P, cause mild, transient pancreatitis; other more virulent mutants, like CB4-V may cause chronic pancreatitis or transient diabetes in mice. Again, this is not a basis for asserting that they are heterologous.

5. The Examiner has made the following statements:

(a) Caggana teaches coxsackievirus CB4-P/CB4-V chimeras, in which an attenuated strain, CB4-P expresses heterologous CB4-V proteins of various types (P1, P2, P3) at various regions of the CB4-P genome, including just downstream from codon 129 of VP1, DE loop (see page 4797- 4798, "Construction of recombinant viruses"; page 4798, Figure 1; pages 4799-4801, bridging paragraph; and page 4802, second column, first line).

(b) The VP1 region encodes capsid, which itself is immunogenic and thus contains epitopes (B-cell and/or T-cell).

(c) Applicant has argued that Caggana's chimerics are not intended to be encompassed by the instant claims because Caggana replaces regions of CB4 viruses with other regions of CB4 viruses. Applicant argues that the replacement of CB4-P genes with CB4-V genes is not a heterologous nucleic acid insertion. While the Office acknowledges that the CB4-P and CB4-V strains of coxsackievirus differ by about five amino acids, they remain structurally distinct strains because they have different amino acid sequences that renders one virulent and the other non-virulent. Even though the virulence is credited to one amino acid residue in the capsid protein of VP1 (Caggana, abstract), the sequences of the two remain different. The VP1 region of CB4-P is not *the same* as the VP1 region of CB4-V structurally (amino acid difference) and functionally (virulent, non-virulent). As such, Caggana's chimeric meets the claim limitations of being a CB4 virion with heterologous nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion, wherein heterologous nucleic acid is defined as "not otherwise naturally present in the genome of the virus". In the instant case, the P1 region of CB4-V was not naturally present in the genome of the CB4-P virus. Therefore, the claims are encompassed by Caggana.

6. The chimeras (=chimerae) to which the Examiner refers (at Para. 5(a), line 1, in the above quote) are merely man-made variants of either strain, any of which might occur given time and correct circumstances; the use of recombinant DNA technology permits immediate analysis instead. Technically speaking, one might refer to such minor changes as intratypic chimeric viruses, to denote the fact that one **homologous** region of a related strain of the same serotype was used to **replace** the original sequence. Thus, whether one mutates a nucleotide to change one amino acid, or one excises the coding sequence for 20 contiguous amino acids and puts in its place the mutated coding sequence in which the same nucleotide has been mutated, the outcome is the same. Viruses of the present claims **are also chimeras** -- characterized in this case by the *insertion* of truly heterologous sequences such as ovalbumin or HIV peptides. The process of cloning described by Caggana involved removal of a CB4 sequence, and re-insertion of another related and closely homologous (which is to say, few differences at the nucleotide level) CB4 sequence into that space - a 'functional sequence replacement'. As the re-inserted similar sequence was from a very closely related CB4 genome with extremely few differences at the nucleotide and amino acid levels, this represents *replacement* of one sequence with another, nearly identical sequence. It is not insertion, as would be the case if the coding sequence for a non-CB protein such as green fluorescent protein (or an HIV peptide) were inserted into the intact CB genome, making such chimeric virus's nucleic acid molecule longer than that of the parental virus.

7. Again, the CB4 nucleic acid sequence (from the -V or -P variant) that replaces the original CB4 sequence in the other virus should not be considered heterologous, *as long as* the replacing sequence is the former sequence's ***homologue*** in the donor genome. The Examiner's extreme interpretation of the definition of "heterologous" used in the application

> "[t]he term 'heterologous polypeptide' refers to a polypeptide which is not otherwise naturally expressed by the virus. The term 'heterologous nucleic acid' refers to any nucleic acid which is not otherwise naturally present in the genome of the virus at the position in which it is inserted"

makes poor biological sense, in my opinion. This is indeed the case in Caggana when replacing the CB4-P protein 1D with the protein 1D from CB4-V). While the Office is correct in stating that "[t]he VP1 region of CB4-P is not *the same* as the VP1 region of CB4-V structurally (amino acid difference) and functionally (virulent, non-virulent)," I disagree with the notion that Caggana's "chimeric" (see my comment above) is "a CB4 virion with **heterologous** nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion." It seems to be a contradiction in terms to view a homologous gene, sequence, or protein as "heterologous." In the case of the viruses described in Caggana, it is clear to me that the replacement sequence is **not heterologous.**

8. To summarize, the cloning strategy described in the present application to create the claimed recombinant viruses and nucleic acid molecules differs fundamentally from that described by Caggana in that, here, truly heterologous sequences (such as ovalbumin, HIV gag p24, proteins that are not found in CB viruses **in nature**[1]), that include additional coding sequence are inserted into CB4 cloning vectors (not merely replacing existing, homologous sequences). Because this is "insertional cloning," the overall coding capacity of the recombinant virus increases significantly compared to that of the "host" CB4 strain's genome. The viruses and the manipulations described in Caggana are distinct from the claimed recombinant chimeric viruses and the methods used to generate them, as described in this application.

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1 or, as stated in the words of the Application's definition of "heterologous": "a polypeptide which is not ... ***naturally*** expressed by the virus..." or "any nucleic acid which is not ... ***naturally*** present in the genome of the virus..." *(emphasis added)*

9. Viruses that are genetically *equivalent* to CB4-P can be manipulated genetically per the application to allow insertions of heterologous nucleic acids in defined spots as described, *e.g.*, to yield an internal fusion of VP1 (see claim 7) or inserted in-frame and directly upstream of VP4 coding sequences (see claim 13). I know of no reason why one could not use, therefore, any strain of CB4 (or any of the 6 CB serotypes), to produce the chimerae described by the Applicants. There is no reason that a skilled person would be limited in his/her ability to practice this invention using CB4-P or any other CB4 virus. Any genetic variant of the JVB strain of the CB4 serotype can be used in the very same way. JVB is publicly available from the ATCC. Thus, the skilled person would not have to resort to any other deposits of CB4-P virus to practice this invention fully. Moreover, there is nothing special about the serotype B4 versus the other five CB serotypes. Once a person skilled in the field has been apprised of the present invention and read the application, that person will be able to practice it as written in the claims. Indeed, what works in CB4-P to create a virus expressing a heterologous polypeptide that can act a as an immunogen would also work in CB4-JVB or CB4-V (notwithstanding other considerations like virulence). Indeed, what works in CB4-P would also work in any strain of virus of the other coxsackievirus B serotypes.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

5-22-05

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1972-1979	Ph.D. Department of Biology, UCSD La Jolla, CA Thesis Advisors: David E. Kohne, Ph.D. and William C. Baxt, M.D.

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Mar 1985-Jun 1985	Visiting Assistant Professor, Department of Microbiology, Health Science Center, University of Texas, San Antonio, TX
Nov 1981-Jun 1985	Assistant Research Microbiologist, School of Public Health University of California, Berkeley, CA
Sep 1980-Nov 1981	Research Associate, Center for Neurologic Study, La Jolla, CA
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Bibliography:

Research papers

1] Allison W, Swain L, Tracy S, Benitez L (1973) Inactivation of lactoperoxidase and the acyl phosphatase activity of oxidized glyceraldehyde-3-phosphate dehydrogenase by phenyl-diimid and phenylhydrazine. Arch Biochim Biophys 155:400-404.

2] Commer P, Schwartz, C, Tracy S, Tamaoki T, Chiu J (1979) Dexamethasone inhibits alpha-fetoprotein gene expression in developing mouse liver. Biochem Biophys Res Comm 89:1294-1299.

3 Tracy S, Kohne D (1980) Detection, sizing, and quantitation of polyadenylated RNA in the nanogram-picogram range. Biochemistry 19:3792-3799.

4] Tracy S (1981) Improved, high-yield methodology for the isolation of nucleic acids from agarose. Prep Biochemistry 11:251-268.

5] Tracy S, Smith R (1981) A comparison of poliovirus genomes by RNA:cDNA hybridization. JGenVirol 55:193-199.

6] Kohne D, Gibbs C, Smith R, Meinke W, Tracy S, White L (1981) Virus detection by nucleic acid hybridization: Examination of normal and amyotrophic lateral sclerosis tissues for the presenceof poliovirus. J Gen Virol 56:223-233.

7] Tracy S (1984) A comparison of genomic homologies among the coxsackievirus B group: Use of fragments of the cloned coxsackievirus B3 genome as probes. J Gen Virol 65:2167-2172.

8] Tracy S (1985) Comparison of genomic homologies in the coxsackievirus B group by use of RNA:cDNA dot blot hybridization. J Clin Microbiol 21:371-374.

9] Tracy S, Chapman N, Liu H (1985) Molecular cloning and partial characterization of the coxsackievirus B3 genome. Arch Virol 85:157-163.

10] Tracy S, Liu H, Chapman N (1985) Coxsackievirus B3: Primary structure of the 5' non-coding and the capsid protein coding regions of the genome. Virus Res 3:263-270.

11] Rotbart H, Levin M, Villareal L, Tracy S, Semler B, Wimmer E (1985) Factors affecting the detection of enteroviruses in cerebrospinal fluid with coxsackievirus B3 and poliovirus type 1 cDNA probes. J Clin Microbiol 22:220-224.

12] Tracy S, Latham A (1986) Rapid immunoprecipitation of coxsackie B viruses and identification by nucleic acid hybridization. Diagnostic Microbiol Infect Dis 4:327-333.

13] Semler B, Johnson V, Tracy S (1986) A molecular recombinant plasmid from cDNA clones of poliovirus and coxsackievirus produces an infectious virus that is temperature sensitive. Proc Natl Acad Sci USA 83:1777-1781.

14] Beck M, Tracy S (1989) Murine cell-mediated immune response recognizes an enterovirus group-specific antigen(s). J Virol 63:4148-4156.

15] Coller B, Chapman N, Beck M, Pallansch M, Gauntt C, Tracy S (1990) Echovirus 22 is an atypical enterovirus. J Virol 64:2692-2701.

16] Chapman N, Tracy S, Gauntt C, Fortmueller U (1990) Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. J Clin Microbiol 28:843-850.

17] Beck M, Chapman N, McManus B, Mullican J, Tracy S (1990) Secondary enterovirus infection in the murine model of myocarditis: Pathologic and immunologic aspects. Am J Pathology 136:669-681.

18] Beck M, Tracy S (1990) Evidence for a group-specific enteroviral antigen(s) recognized by human T cells. J Clin Microbiol 28:1822-1827.

19] Okada I, Matsumori A, Kawai C, Yodoi J, Tracy S (1990) The viral genome in experimental murine coxsackievirus B3 myocarditis: A northern blotting analysis. J Molec Cell Cardiol 22:999-1008.

20] Wiegand V, Tracy S, Chapman N, Wucherpfennig C (1990) Enteroviral infection in end-stage dilated cardiomyopathy. Klin Wochenschr 68:914-920.

21] Tracy S, Wiegand V, McManus B, Gauntt C, Pallansch M, Beck, M, Chapman N (1990) Molecular approaches to enteroviral diagnosis in idiopathic cardiomyopathy and myocarditis. J Am Coll Cardiol 15:1688-1694.

22] Tracy S, Chapman N, McManus B, Pallansch M, Beck M, Carstens J (1990) A molecular and serologic evaluation of enteroviral involvement in human myocarditis. J Molec Cell Cardiol 22:403-414.

23] Coller B, Tracy S, Etchison D (1991) Cellular protein p220 is not cleaved during echovirus 22 replication. J Virol 65:3903-3905.

24] Gauntt C, Lutton C, Arizpe H, Higdon A, Tracy S (1991) Autoimmune reactions in coxsackievirus B3-induced

murine myocarditis. Allergy Immunol 10:23-31.

25] Dasmahapatra B, Rozhon E, Hart A, Cox S, Tracy S, Schwartz J (1991) Cell-free expression of the coxsackievirus 3C protease using the translational initiation signal of an insect virus RNA and its characterization. Virus Res 20:237-249.

26] Beck M, Tracy S, Coller B, Chapman N, Johnson J, Lomonossoff G (1992) Comoviruses and enteroviruses share a T cell epitope. Virology 186:238-246.

27] Tracy S, Chapman N, Tu Z (1992) Coxsackievirus B3 from an infectious cDNA copy of the genome is cardiovirulent in mice. Arch Virol 122:399-409.

28] Kyu B, Matsumori A, Sato Y, Okada I, Kawai C, Chapman N, Tracy S (1992) Cardiac persistence of cardioviral RNA detected by the polymerase chain reaction in a murine model of dilated cardiomyopathy. Circulation 86(2):522-530.

29] Carstens J, Tracy S, Chapman N, Gauntt C (1992) Detection of enteroviruses in cell cultures by using in situ transcription. J Clin Microbiol 30:25-35.

30] Gauntt C, Higdon A, Arizpe H, Tamayo M, Crawley R, Henkel R, Pereira M, Tracy S, Cunningham M (1993) Epitopes shared between coxsackievirus B3 (CVB3) and normal heart tissue contribute to CVB3-induced murine myocarditis. Clin Immun Immunopathol 68:129-134.

31] Sato S, Burke A, Benson W, Mergner W, Tracy S, Gauntt C, Virmani R (1993) The pathology of murine coxsackievirus B3 myocarditis: an in situ hybridization study. Cardiovasc Pathol 2:107-115.

32] Chapman N, Tu Z, Tracy S, Gauntt C (1994) An infectious cDNA copy of the genome of a non-cardiovirulent coxsackievirus B3 strain: Its complete sequence analysis and comparison to the genomes of cardiovirulent coxsackieviruses. Arch Virol 135:115-130.

33] Tu Z, Chapman N, Hufnagel G, Tracy S, Romero J, Barry W, Zhao L, Currey K, Shapiro B (1995) The cardiovirulent phenotype of coxsackievirus B3 is determined at a single site in the genomic 5' non-translated region. J Virol 69:4607-4618.

34] Harrison C, Britt W, Chapman N, Mullican J, Tracy S (1995) Effects of immunization with glycoprotein B of guinea pig cytomegalovirus (gpCMV) on maternal immunity and congenital infection rates after maternal primary gestational gpCMV infection. J Infect Dis 172:1212-1220.

35] Chapman N, Romero J, Pallansch M, Tracy S (1997) Sites other than nucleotide 234 determine cardiovirulence in natural isolates of coxsackievirus B3. J Med Virol 52:258-261.

36] Lee C, Maull E, Chapman N, Tracy S, Gauntt C (1997) Genomic regions of coxsackievirus B3 associated with cardiovirulence. J Med Virol 52:341-347.

37] Lee C, Maull E, Chapman N, Tracy S, Wood J, Gauntt C (1997) Generation of an infectious cDNA of a highly cardiovirulent coxsackievirus B3 (CVB3m) and comparison to other infectious CVB3 cDNAs. Virus Res 50:225-235.

38] Carson S, Chapman N, Tracy S (1997) Purification of the putative coxsackievirus B receptor from HeLa cells. Biochem Biophys Res Comm 233:325-328.

39] Carson S, Hobbs JT, Tracy S, Chapman NM (1999) Expression of the coxsackievirus and adenovirus receptor in cultured human umbilical vein endothelial cells: regulation in response to cell density. J Virol 73:7077-7079.

40] Tracy S, Hofling K, Pirruccello S, Lane PH, Reyna SM, Gauntt CJ (2000) Group B coxsackievirus myocarditis and pancreatitis in mice: Connection between viral virulence phenotypes. J Med Virol 62:70-81.

41] Chapman NM, Ragland A, Leser JS, Hofling K, Semler BL, Tracy S (2000) A group B coxsackievirus/poliovirus 5' non-translated region chimera serves as an attenuated vaccine strain in mice. J Virol 74:4047-4056.

42] Dunn JJ, Chapman NM, Tracy S, Romero JR (2000) Natural genetics of cardiovirulence in coxsackievirus B3 clinical isolates: Localization to the 5' non-translated region. J Virol 74:4787-4794.

43] Chapman NM, Kim K-S, Tracy, S, Jackson J, Hofling K, Leser JS (2000) Coxsackievirus expression of the murine secretory protein IL-4 induces increased synthesis of IgG1 in mice. J Virol. 74:7952-7962.

44] Hofling K, Tracy S, Chapman NM, Kim K-S, Leser JS (2000) Expression of an antigenic adenovirus epitope in a group B coxsackievirus. J Virol 74:4570-4578.

45] Willian S, Tracy S, Chapman NM, Leser JS, Romero JR, Shapiro B, Currey K (2000) Mutations in a conserved enteroviral RNA oligonucleotide sequence affect positive strand RNA synthesis. Arch Virol. 145:2061-2086.

46] Tracy S, Drescher KM, Chapman NM, Kim K-S, Carson SD, Pirruccello S, Lane PH, Romero JR, and Leser JS (2002) Toward testing the hypothesis that group B coxsackieviruses (CVB) trigger insulin-dependent

diabetes: Inoculating NOD mice with CVB markedly lowers diabetes incidence. J Virol 76:12097-12111.

47] Dunn J, Bradrick S, Chapman N, Tracy S, Romero J (2003) The stem loop II within the 5' nontranslated region of clinical coxsackievirus B3 genomes determines cardiovirulence phenotype in a murine model. J Infect Dis.187:1552-61.

48] Drescher KM, Kono K, Bopegamage S, Carson SD, Tracy S (2004) Coxsackievirus B3 infection and type 1 diabetes development in NOD mice: Insulitis determines susceptibility of pancreatic islets to virus infection. Virology 329:381-394.

49] Drescher KM, Kocakova P, Chapman NM, Tracy S. (2004) CVB-based vaccines alter immune responses to self in virus-infected mice. Immunology.

50] Lee CK, Kono K, Haas E, Kim KS, Drescher KM, Chapman NM, Tracy S. (2005) Characterization of an infectious cDNA copy of the genome of a naturally-occurring, avirulent coxsackievirus B3 clinical isolate. J General Virology 86:197-210

51] Tracy S, Drescher KM, Chapman N, Kono K, Tapprich W (2005, *in press*) Evolution of virulence in picornaviruses. Curr Topics Microbiol Immunol "Quasispecies: Concept and implications for virology" (editor: E. Domingo)

52] Kim K-S, Tracy S, Tapprich W, Bailey J, Lee C-K, Kim K, Barry W, Chapman N. (2005, *in press*) 5' Terminal deletions occur in coxsackievirus B3 during replication in murine hearts and cardiac myocyte cultures and correlate with encapsidation of negative-strand viral RNA. J Virology.

Review Articles

1] Tracy S, Chapman N, Beck M (1991) Molecular biology and pathogenesis of coxsackie B viruses. Rev Med Virol 1:145-154.

2] Tracy S, Hufnagel G, Chapman N (1992) Interesting problems in enteroviral inflammatory heart disease. Herz 17:79-84.

3] Tracy S, Chapman N, Romero J, Ramsingh A (1996) Coxsackievirus B3 cardiovirulence determinants and their role in inflammatory heart muscle disease. Trends Microbiol 4:175-178.

4] Ramsingh A, Chapman N, Tracy S (1997) Coxsackieviruses and diabetes. BioEssays 19:793-800.

5] Kim K-S, Hufnagel G, Chapman NM, Tracy S (2001) Group B coxsackieviruses and myocarditis. Rev Med Virol. 11:355-368

Book Chapters

1] Tracy S (1985) Aspects of using nucleic acid filter hybridization to characterize and detect enteroviral RNAs, pp. 109-125. In: Proceedings of the International Symposium for the Detection of Infectious Viruses and Microorganisms (Berkeley, 1983). D Kingsbury, S Falkow, eds.; Academic Press, New York.

2] Tracy S (1988) The organization of the coxsackievirus genome, pp. 19-34. In: The Coxsackieviruses - An Update. H Friedman, M Bendinelli, eds.; Plenum Press, New York.

3] Gauntt C, Godeny E, Lutton C, Arizpe H, Chapman N, Tracy S, Revtyak, G, Valente A, Rozek M (1989) Mechanism(s) of coxsackievirus-induced acute myocarditis in the mouse, pp. 161-182. In: Medical Virology VIII. L de la Maza, E Peterson, eds.; Lawrence Erlbaum Assoc., Inc: Hillsdale, NJ.

4] Tracy S, Chapman N, Pistillo J (1992) Detection of enteroviruses by PCR. In: Frontiers of Virology (Vol. 1): Diagnosis of Human Viruses by Polymerase Chain Reaction (PCR) Technology, pp. 331-344. Y Becker, G Darai, eds.; Springer-Verlag, Berlin.

5] Gauntt C, Higdon A, Arizpe H, Maull E, Lutton C, Beck M, Chapman N, McManus B, Mullican J, Tracy S (1993) Specific and non-specific heart defenses in enteroviral infections. In: Idiopathic Dilated Cardiomyopathy, H Figulla *et al.*, eds.; Springer-Verlag, Berlin.

6] Tracy S, Chapman N, Rubocki R, Beck M (1995) The host immune response to enterovirus infections. Human Enterovirus Infections, H. Rotbart, ed.; ASM Press, New York.

7 Romero J, Chapman N, Rotbart H, Tracy S (1996) Polymerase chain reaction detection of the human enteroviruses. In: PCR: Protocols for the Diagnosis of Human and Animal Virus Diseases, pp. 175-193. Y Becker, G Darai, eds.; Springer-Verlag, Berlin.

8] Chapman N, Ramsingh A, Tracy S (1997) The genetics of CVB virulence, pp.227-258. In: The Coxsackie B Viruses. Current Topics Microbiol Immunol 223. S Tracy, N Chapman, B Mahy, eds.; Springer Verlag, Heidelberg.

9] Chapman N, Gauntt C, Tracy S (1999) Enteroviruses. In: The Embryonic Encyclopedia of Life Sciences. Nature Publishing Group; London.
10] Chapman N, Tracy S (1999) Enteroviruses. In: Encyclopedia of Microbiology, 2nd. Edition. J. Lederberg *et al.*, eds.; Academic Press, San Diego.
11] Chapman N, Gauntt C, Tracy S (2002) Immune responses to coxsackievirus infections. In: The Picornaviruses. B Semler, E Wimmer, eds.; Plenum Press, New York.
12] Kim K-S, Hofling K, Chapman NM, Tracy S (2002) Cardiotropic viruses. In: Myocarditis. Cooper LS, Knowlton K, eds.; Mayo Academic Press, Rochester MN.
13] Chapman N, Kim K-S, Tracy S (2003) The group B coxsackieviruses as vaccines and vectors. In: Cardiomyopathy and Heart Failure. Matsumori A, ed. Kluwer Academic Publishers, Norwell MA.

Volumes Edited

1] Tracy S, Chapman N, Mahy B, eds. (1997) The Coxsackie B Viruses. Current Topics in Microbiology and Immunology, vol. 223. Springer-Verlag, Heidelberg.

Electronic Publications

1] Chapman N, Gauntt C, Tracy S (1999) Enteroviruses. In: The Embryonic Encyclopedia of Life Sciences. Nature Publishing Group; London. http://www.els.net
2] Tracy S, Chapman N (2001) Enteroviruses. In: The Springer Index of Viruses. Springer-Verlag, C. Tidona, G. Darai, eds. http://oesys.springer.de/viruses
3] Kim K-S, Chapman N, Tracy S (2005) Enteroviruses. In: The Embryonic Encyclopedia of Life Sciences. Nature Publishing Group; London. http://www.els.net

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PATENT TRADEMARK OFFICE

DECLARATION OF BARBARA WEISER, M.D. PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
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Dear Sir:

I, the undersigned, declare as follows:

1. I am an expert in the field of molecular virology and clinical infectious disease, with an emphasis on HIV. I have worked with various different classes of viruses for about 25 years and have focused my research on the RNA virus HIV-1 for the past 21 years. I am an author/co-author of about 60 peer-reviewed publications, review articles and book chapters in this field. My *Curriculum Vitae* is attached. I am currently a Co-Director of HIV Research at the Wadsworth Center, New York State Department of Health, and a Professor of Medicine, Division of HIV Medicine, Albany Medical College. I have been an active member of the NIH Women's Interagency HIV Study (*WIHS*) Laboratory Committee since 1993 and have chaired the subcommittees on Virology, and HIV Infection in Drug-Using Women. I am currently a member of the *WIHS* Scientific Advisory Board and chair of its Scientific Working Group on HIV Virology. I am familiar with the research of Dr. Arlene Ramsingh, co-inventor of the above-identified patent application (particularly as it concerns potential HIV immunogens) as we been staff members in the same institution for the past 14 years.

2. I have reviewed the relevant sections of the Office Action and the patent application, including the claims. My comments below are based on my specialized knowledge of RNA viruses and their particular properties that distinguish them from DNA viruses and from prokaryotic and eukaryotic cells as concerns their nucleic acids, replication and unique structural variation and heterogeneity. Although I am not a specific expert in coxsackieviruses, I do know a great deal about how they and other RNA viruses resemble and also differ in significant ways from HIV-1. I note that the present invention does focus to a large degree on production and use of recombinant CB4 viruses into which have been inserted heterologous nucleotide sequences that encode HIV peptides for use as immunogens and vaccines.

3. My research focuses on HIV-1 sequence variation and its impact on pathogenesis and treatment of HIV and AIDS. HIV-1 variation is ubiquitous and occurs not only among infected individuals, but also among HIV-1 strains or "quasispecies" obtained from different organs in the same person. HIV-1 envelope genes isolated from people in Africa and New York are highly divergent and may differ by ~25-30% of their sequences. HIV-1 and coxsackie genomes diverge from one another by much more than that, of course. It is highly probable that the diversity of HIV-1 sequences among virions within the body of a single patient across various compartments (blood, lymph, lymphatic tissue, mucosal tissue of the gut and reproductive system, *etc.*) is greater than the sum total of diversity of all CB4 viruses throughout the world. That fact has been proven to be true when comparing HIV-1 diversity in one patient with the influenza virus worldwide. Having said that, it is important to appreciate for the present discussion that the genes and their polypeptide products in all these HIV-1 variants (some differing quite extensively, *e.g.*, by 25-30% of the total genome) are nonetheless **not considered to be "heterologous"** one to the other.

4. In view of the above, I was rather stunned to learn that the Patent Office, when considering the publication by Caggana *et al. J Virol.* (1993) *67*:4797-803) and applying it to the invention being claimed in the above-identified application, took the position that the CB4-P and CB4-V variants were heterologous to one another. That is a view that would simply not be accepted by any virologist (or other biologist) for reasons presented below. In comparing these two variants over a stretch of close to 3300 nt's (including the most variable part of the viral genome), only 9

total nucleotides differed, of which only 5 were in coding regions, resulting in viruses that differed in a grand total of 5 amino acids in three viral proteins. A single amino acid position in the VP1 protein was shown to be responsible for the phenotype of interest (virulence in mice). This amount of variation is exceedingly small and is nowhere near the extent that would be considered as defining separate viral serotypes or strains or classes or whatever other taxonomic term one chooses, and would certainly not be considered by people skilled in this field as "heterologous". Indeed, as I relate below in Sec. 7, major "functional" differences in HIV-1 pathogenicity that are associated with even greater nucleotide sequence differences than those which distinguish CB4-V from CB4-P, are not considered heterologous by anyone's definition of the term.

5. Another reason why the "new" CB4 nucleic acid sequence (CB4-V), described in the Caggana paper and replacing the original CB4 sequence in the CB4-P variant, cannot fairly be considered heterologous is because these sequences are, in fact, **homologous:** they encode the same protein with the same biological function, but with one mutated amino acid. Something that is, in fact, homologous cannot at the same time be heterologous. That situation is completely distinct from this invention - where sequences truly foreign to CB4 viruses are inserted in certain sites to be appropriately expressed so that these recombinant virions can serve as immunogens to evoke immune responses to these non-coxsackievirus peptides.6. The Examiner has focused on this minimal CB4-V - CB4-P difference in conjunction with the definition for "heterologous" used in the application and asserted first that:

> Even though the virulence is credited to one amino acid residue in the capsid protein of VP1 ..., the sequences of the two remain different. The VP1 region of CB4-P is not ***the same as*** the VP1 region of CB4-V structurally (amino acid difference) and functionally (virulent, non-virulent).

(emphasis added). Those statements are accurate and correct. From this, the Examiner went on to characterize the virus described in the Caggana paper as having

> ...**heterologous** nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion, wherein heterologous nucleic acid is defined as "not otherwise naturally present in the genome of the virus". ... the P1 region of CB4-V was not naturally present in the genome of the CB4-P virus.

The Examiner's interpretation of the definition of "heterologous" used in the application is strained, and not in line with how those in the field view RNA viruses, their nucleic acids, and their proteins, the variation in the sequences of these molecules, and their relationship with one another. It is these relationships that are at the heart of the concepts of 'homologous' and "heterologous' (because something is homologous or heterologous only to a reference sequence or virus or bacterium or animal species). Thus, I must firmly disagree with the Patent Office's assertion that the virus described in Caggana is

> "a CB4 virion with heterologous nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion".

Rather, it is a CB4 virus in which a segment of a nucleic acid (a viral gene) has been replaced with the **homologous** (albeit non-identical) segment from a very closely related virus of the same taxon. Calling it heterologous is the polar opposite of what I and scientists skilled in this field would call it, the application's definition notwithstanding. Reading the application alone, it is unequivocal to me that the Applicants did not intend to include within their definition of "heterologous polypeptides" other coxsackievirus polypeptides, and certainly not *homologous* CB4 polypeptides as in Caggana.

7. It may be instructive to compare the situation of the CB4-P and CB4-V viruses of Caggana with differences between HIV-1 viruses where defined sequence differences are associated with different levels of pathogenicity. In studying determinants of HIV-1 attenuation and pathogenesis, we and others have turned our attention to "long term non-progressors" (LTNP), patients infected with the virus for say ten or more year but in whom (a) the disease does not progress and (b) there is no significant depletion of CD4+ T cells. We reported on an individual[1] who underwent a transition from LTNP to rapidly progressive infection and analyzed hundreds of clones of full-length HIV-1 RNA genomes from the plasma of this individual before and during the transition. We found that a 20 bp deletion appeared to confer attenuation on the virus. When the infection became progressive, all viruses had intact (non-deleted) sequences and were derived from

[1] Fang G, Burger H, Chappey C, Rowland-Jones S, Visosky A, Chen C, Moran T, Townsend L, Murray M, and Weiser B. (2001) Analysis of transition from long-term non-progressive to progressive infection identifies sequences which may attenuate HIV-1, *AIDS Res and Hum Retroviruses*, 17:1395-1404

a minor species present earlier. Comparing this to the CB4-V and CB4-P viruses described in Caggana, one sees that a defined molecular change, somewhat larger in magnitude in our case, was responsible for the virus's pathogenicity. However, the sequence with the 20 bp deletion was not "heterologous" to the intact (non-deleted) sequence and vice versa. All the sequences present in the subject were derived from a common ancestor. Employing the application's definition here, the nucleic acid lacking the 20 bp was "not naturally present in the genome" of other viruses without the deletion. The nucleic acid of the virulent form of HIV-1 in this subject "was not naturally present" in the deleted nucleic acid of the attenuated variant. Yet, it would have been considered absurd had we labeled these sequences as heterologous. Similarly, the Patent Office's calling the CB4-P and CB4-V viruses heterologous is scientifically totally untenable.

8. In contrast to the discussion in Section 7, above, examples of sequences from related viruses that are nonetheless considered heterologous to HIV-1 are sequences from the viruses HIV-2 and SIV (simian immunodeficiency virus). Both HIV-2 and SIV are considered by the virology community to be distinct viruses from HIV-1 and vary from each other by more than 35% at the nucleotide level. Different primers are needed to amplify these different genomes. The chimeric virus composed of portions of HIV-1 and SIV, known now as the SHIV (engineered by Sodroski and colleagues for use primarily in vaccine design), expresses the *env* gene of HIV-1 yet can still infect and replicate in rhesus macaques (the natural host of SIV but not of HIV-1). This recombinant, chimeric virus, ***like*** **the recombinant chimeric coxsackieviruses** of the above-identified invention, but ***unlike*** **the CB4-V and CB4-P** viruses of the Caggana reference, is definitely considered to contain heterologous sequences.

9. In summary, I reiterate that the Patent Office has adopted here a meaning of ***heterologous*** polypeptides and nucleic acid sequences that is fundamentally different from the meaning accepted by the scientific community and from that in the application. The Caggana reference describes replacement of a few, subtly varying homologous nucleotide sequences in the laboratory generation of an attenuated CB4 variant from an extremely closely related virulent CB4 variant. Based upon my knowledge of an extensive literature dealing with viral variation and my expertise in this very field, I believe that no virologist would consider the altered sequences

described in the Caggana reference to be heterologous. Considering both the present patent application and what is accepted in the field, I can see no proper basis for the Patent Office's conclusions about what this reference says. Likewise, I do not see any connection between the reference and the Applicants' claims.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

5/25/05 Barbara Weiser

Date Barbara Weiser

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Date Barbara Weiser

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Education

Vassar College, A.B. in English, 1969
SUNY Stony Brook, graduate study in Cell Biology, 1970-71
University of Pittsburgh School of Medicine, M.D., 1975

Postdoctoral Training

Intern and Resident in Internal Medicine, Bellevue Hospital-New York University Medical Center, 1975-78
Infectious Diseases Fellow, Memorial Sloan-Kettering Cancer Center, 1978-80
Postdoctoral Research Fellow, Laboratory of Dr. Harold Varmus, Department of Microbiology, University of California at San Francisco, 1980-83
Postdoctoral Research Fellow, Laboratory of Dr. William Robinson, Infectious Disease Division, Department of Medicine, Stanford University, 1984-85

Staff Appointments

Instructor of Medicine, Cornell University Medical College, Khymer Refugee Project, Khao-I-Dang Holding Center, Thailand, June-July, 1980
Assistant Professor of Medicine, Division of Infectious Diseases, San Francisco General Hospital, University of California at San Francisco, January, 1981
Assistant Professor of Medicine and Microbiology, SUNY Stony Brook, 1985-91
Co-Director of HIV Research, Wadsworth Center, New York State Department of Health, 1991-present
Associate Professor of Medicine, Divisions of HIV Medicine and Infectious Diseases, Albany Medical College, 1991-1998
Professor of Medicine, Albany Medical College, 1998-present

Board Certification

Diplomate, American Board of Internal Medicine, 1978

Honors

American Cancer Society Postdoctoral Research Fellowship, 1981-1982
National Heart, Lung and Blood Institute Clinical Investigator Award, 1982-1987
Mary Pangborn Award for Research with an Impact on Clinical Medicine, New York State Department of Health, 1997

Patent

US Patent: "Analysis of HIV-1 Co-Receptor Utilization in the Clinical Care of HIV-1-Infected Patients," patent number US 09/963,064," 2003

Present National Committee Membership

NIH Women's Interagency HIV Study (WIHS) Laboratory Committee, 1993-present (Chair of Subcommittees on Virology, 1994-98, and HIV Infection in Drug-Using Women, 1995-98)
WIHS Scientific Advisory Board, 2002-present
Chair, WIHS Scientific Working Group on HIV Virology, 2002-present

Present Local Committee Membership

Wadsworth Center Biosafety Committee, 1995 - present

Past Committee Membership

National Institutes of Health Committees
AIDS Clinical Trial Group (ACTG) Basic Research Committee, 1987-90
ACTG Virology Committee, 1987-90
AIDS Related Research Ad Hoc Study Sections, 1989-91 and 95-96
NIAID AIDS Research Review Committee, Basic Science I, 1991-1994
WIHS Review Team, 1995-1996
WIHS Executive Committee, 1997-2002

Institutional Committees
SUNY Stony Brook Biomedical Research Support Committee, 1988-91
SUNY Stony Brook Biotechnology Center Committee, 1988-91
Wadsworth Center Tuberculosis Committee, 1992
Wadsworth Center Axelrod Institute Space Allocation Committee, 1993
Wadsworth Center Axelrod Symposium Committee, 1994
Wadsworth Center Peer Review Board, 1995-1998
Chairperson, Albany Medical Center Search Committee for the Chief of the Division of HIV Medicine, 1999-2000

Membership in Professional Societies

American Society for Microbiology
American Association for the Advancement of Science
Infectious Diseases Society of America
HIV Medicine Association
International AIDS Society
New York Society of Infectious Diseases

Conference Organizer

National Conferences on Women and HIV, Los Angeles, 1997 and 1999
Satellite Meeting on HIV Infection in Women, 4th Conference on Retroviruses and Opportunistic Infections, Washington, DC, 1997

Ad Hoc Reviewer

Virology
AIDS
Journal of Virology
Clinical Infectious Diseases
Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology

BIBLIOGRAPHY

Doerfler W, Burger H, Ortin J, Fanning E, Brown DT, Westphal M, Winterhoff U, Weiser B, and Shick J. (1974) Integration of adenovirus DNA into the host genome. Cold Spring Harbor Symposium on Quantitative Biology 39:505-521.

Weiser B, Lange M, Fialk M, Singer C, Szatrowski T, and Armstrong D. (1981) Prophylactic trimethoprim-sulfamethoxazole during consolidation chemotherapy for acute leukemia: a controlled trial. *Ann Intern Med* 95:436-438.

Arena FP, Perlin M, Brahman H, Weiser B, Armstrong D. (1981) Fever, rash, and myalgias of disseminated candidiasis during antifungal therapy. *Arch Intern Med* 141:1233.

Ganem D, Weiser B, Barchuk A, Brown J, and Varmus HE. (1982) Biological characterization of acute infection with ground squirrel hepatitis virus. *J Virol* 44:366-373.

Weiser B, Ganem D, Seeger C, and Varmus HE. (1983) Closed circular viral DNA and asymmetrical heterogeneous forms in livers from animals infected with ground squirrel hepatitis virus. *J Virol* 48:1-9.

Burger H, Weiser B, Robinson WS, Lifson J, Engleman E, Rouzioux C, Brun-Vézinet F, Barré-Sinoussi F, Montagnier L, and Chermann J-C. (1985) Transient antibody to LAV/HTLV III and T lymphocyte abnormalities in the wife of a man who developed AIDS. *Ann Intern Med* 103:545-547.

Burger H, Weiser B, Robinson WS, Lifson J, Engleman E, Rouzioux C, Brun-Vézinet F, Barré-Sinoussi F, Montagnier L, and Chermann J-C. (1986) Transmission of LAV/HTLV III in sexual partners: seropositivity does not predict infectivity in all cases. *Am J Med* 81:5-10.

Weiser B, Burger H, Steimer K, Lifson J, Engleman E, Grimson R, and Robinson WS. (1987) Antibody to human immunodeficiency virus correlates with decreased T helper lymphocytes in asymptomatic individuals. *J Med Virol* 23:237-244.

Burger H, Paul D, Wendel I, Neff S, Eilbott D, Siegal FP, Gehan K, Grimson R, and Weiser B. (1988) Comparison of antigen immunoassay and reverse transcriptase assay for monitoring HIV infection in an antiviral trial. *J Clin Micro* 26:1890-1892.

Burger H, Peress N, Eilbott D, La Neve D, Orenstein J, Gendelman H, Seidman R, and Weiser B. (1989) HIV in AIDS myelopathy. In Vaccines '89, Modern Approaches to New Vaccines Including Prevention of AIDS. RA Lerner, H Ginsberg, RM Chanock, and F Brown, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 125-131.

Eilbott DJ, Peress N, Burger H, La Neve D, Orenstein J, Gendelman H, Seidman R, and Weiser B. (1989) Human immunodeficiency virus type 1 in spinal cords of acquired immunodeficiency syndrome patients with myelopathy: expression and replication in macrophages. *Proc Natl Acad Sci* USA 86:3337-3341.

Gendelman HE, Orenstein J, Baca LM, Weiser B, Burger H, Kalter DC, and Meltzer MS. (1989) The macrophage in the persistence and pathogenesis of HIV infection. *AIDS* 3:475-495.

Weiser B, Burger H, Eilbott D, Flaherty K, Gulla J, Neff S, Gehan K, Davidson B, Anand R, and Siegal FP. (1989) Efficacy of rifabutin in the treatment of AIDS-related complex. *AIDS* 3:823-827.

Weiser B, Peress N, Eilbott DJ, La Neve D, Seidman R, and Burger H. (1990) Human immunodeficiency virus type 1 expression in the central nervous system correlates directly with extent of disease. *Proc Natl Acad Sci* USA 87:3997-4001.

Siegal FP, Eilbott DJ, Burger H, Gehan K, Davidson B, Kaell AT, and Weiser B. (1990) Dose-limiting toxicity of rifabutin in AIDS-related complex: syndrome of arthralgia/arthritis. *AIDS* 4:433-441.

Burger H, Belman AL, Grimson R, Kaell A, Flaherty K, Gulla J, Gibbs R, Nguyen PN, and Weiser B. (1990) Long HIV-1 incubation periods and dynamics of transmission within a family. *Lancet* 336:134-136.

Burger H, Gibbs R, Nguyen PN, Flaherty K, Gulla J, Belman A, and Weiser B. (1990) HIV-1 transmission within a family: generation of viral heterogeneity correlates with duration of infection. In Vaccines '90, Modern Approaches to New Vaccines Including Prevention of AIDS. RA Lerner, H Ginsberg, RM Chanock, and F Brown, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 255-262.

Cohen A, Weiser B, Afzal Q, and Fuhrer J. (1990) Ventricular tachycardia in two patients with AIDS receiving ganciclovir (DHPG). *AIDS* 4:807-809.

Burger H, Weiser B, Flaherty K, Gulla J, Nguyen P, and Gibbs RA. (1991) Evolution of human immunodeficiency virus type 1 nucleotide sequence diversity among close contacts. *Proc Natl Acad Sci* USA 88:11236-11240.

Weiser B, Nachman S, Burger H, Hsu JH, and Gibbs R. (1993) Serial HIV sequences from a pregnant women and her vertically infected twins suggest one twin was infected in utero and one at birth. In Vaccines '93, Modern Approaches to New Vaccines Including Prevention of AIDS. RA Lerner, H Ginsberg, RM Chanock, and F Brown, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 131-136.

Haigwood NL, Weiser B, Mason BO, Higgins D, and Burger H. (1993) HIV-1 sequence diversity varies with degree of immune deficiency. In Vaccines '93, Modern Approaches to New Vaccines Including Prevention of AIDS. RA Lerner, H Ginsberg, RM Chanock, and F Brown eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 233-242.

Haigwood NL, Weiser B, and Burger H. (1993) Immunotherapeutic strategies for treatment of lentivirus disease. In HIV Molecular Organization, Pathogenicity and Treatment. WJW Morrow and NL Haigwood, eds. Elsevier Science, New York, pp.329-363.

Weiser B, Nachman S, Tropper P, Viscosi KH, Grimson R, Baxter G, Fang G, Reyelt C, Hutcheon N, and Burger H. (1994) Quantitation of human immunodeficiency virus type 1 during pregnancy: relationship of viral titer to mother-to-child transmission and stability of viral load. *Proc Natl Acad Sci* USA 91:8037-8041.

Fang G, Weiser B, Tropper P, Nachman S, Viscosi KH, Moore R, Grimson R, Baxter G, Sporysz N, Melendez M, Reyelt C, Hutcheon N, and Burger H. (1995) Maternal HIV-1 plasma RNA level determined by RT QC-PCR: A major determinant of mother-to-child transmission. In Vaccines '95, Molecular Approaches to the Control of Infectious Diseases. RM Chanock, F Brown, H Ginsberg and E Norrby, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 189-194.

Fang G, Burger H, Grimson R, Tropper P, Nachman S, Mayers D, Weislow O, Moore R, Reyelt C, Hutcheon N, Baker D, and Weiser B. (1995) Maternal plasma human immunodeficiency virus type 1 RNA level: a determinant and projected threshold for mother-to-child transmission. *Proc Natl Acad Sci* USA 92:12100-12104.

Siegal FP, Weiser B, Burger H, Gehan K, Gold JWM, Wynne BA, and Nightingale S. (1995) The development of rifabutin for prophylaxis against Mycobacterium avium complex infections. *European Review of Respiratory Diseases* 5:98-101.

Fang G, Weiser B, Visosky AA, Townsend L, and Burger H. (1996) Molecular cloning of full-length HIV-1 genomes directly from plasma viral RNA. *J Acquir Immune Defic Syndr Hum Retrovirol* 12:352-357.

Weiser B, Burger H, Campbell P, Donelan S, and Mladenovic J. (1996) HIV-1 RNA expression in bone marrows of patients with a spectrum of disease. *AIDS Res and Hum Retroviruses* 12:1551-1558.

Landay A, and Weiser B. (1997) Report from the 4th Conference on Retroviruses & Opportunistic Infections. JAMA web site, http://www.ama-assn.org/special/hiv/ basic.htm.

Burger H, Kovacs A, Weiser B, Grimson R, Nachman S, Tropper P, van Bennekum AM, Elie MC, and Blaner WC. (1997) Maternal serum vitamin A levels are not associated with mother-to-child transmission of HIV-1 in the United States. *J Acquir Immune Defic Syndr Hum Retrovirol* 14:321-326.

Fang G, Siegal FP, Weiser B, Grimson R, Anastos K, Back S, and Burger H. (1997) Measurement of serum HIV-1 RNA by RT QC-PCR distinguishes progressive from non-progressive HIV-1 infection in men and women. *Clin Infect Dis* 25:332-333.

Burger H and Weiser B. (1997) Biology of HIV-1 in women and men. In Obstetrics and Gynecology Clinics of North America H. Minkoff, ed. WB Saunders, Philadelphia, PA, 24:731-742.

Lew J, Reichelderfer P, Fowler M, Bremer J, Carroll R, Cassol S, Chernoff D, Coombs R, Cronin M, Dickover R, Fiscus S, Herman S, Jackson B, Kornegay J, Kovacs A, McIntosh K, Meyer W, Michael N, Mofenson L, Moye J, Quinn T, Robb M, Vahey M, Weiser B, Yeghiazarian T, and the Tube Meeting Workshop Attendees (1998) Determinations of levels of HIV-1 RNA in plasma: reassessment of parameters affecting assay outcome. *J Clin Micro* 36:1471-1479.

Fang G, Zhu G, Burger H, Keithly JS, and Weiser B. (1998) Minimizing DNA recombination during long RT-PCR. *J Virol Methods* 76:139-148.

Fang G, Weiser B, Visosky A, Moran T, Burger H. (1999) PCR-mediated recombination: a general method applied to construct chimeric infectious molecular clones of plasma-derived HIV-1 RNA. *Nature Medicine* 5:239-242.

Philpott S, Burger H, Charbonneau T, Grimson R, Vermund S, Visosky A, Nachman S, Kovacs A, Tropper P, Frey H, Weiser B. (1999) CCR-5 genotype and resistance to vertical transmission of HIV-1. *J Acquir Immune Defic Syndr Hum Retrovirol* 21:189-193.

Weiser B and Burger H. (1999) Commentary on "Immunologic and Virologic Status of the Sydney Blood Bank Cohort" JAMA AIDS/HIV web site:http://www.ama-ssn.org/special/hiv/library/scan/june99/hivcom45.htm

Anastos K, Kalish LA, Hessol N, Weiser B, Melnick S, Burns D, Delapenha R, DeHovitz J, Cohen M, Meyer W, Bremer J, and Kovacs A. (1999) The relative value of CD4 cell counts and quantitative HIV-1 RNA in predicting survival in HIV-1 infected women: results of the Women's Interagency HIV Study (WIHS). *AIDS* 13:1717-1726.

Anastos K, Gange SJ, Lau B, Weiser B, Detels R, Giorgi JV, Margolick JB, Cohen M, Phair J, Melnick S, Rinaldo CR, Kovacs A, Levine A, Landesman S, Young M, Muñoz A, and Greenblatt RM. (2000) The association of race and gender with HIV-1 RNA levels and immunologic progression. *J Acquir Immune Defic Syndr Hum Retrovirol*, 24:218-226.

Burger H and Weiser B. (2001) Biology of HIV-1 in Women and Men. In Obstetrics and Gynecology of Northern America H Minkoff, ed. WB Saunders, Philadelphia, PA, 44:137-143.

Philpott S, Weiser B, Anastos K, Kitchen CMR, Robison E, Meyer WA, Sacks HS, Mathur-Wagh U, Brunner C, and Burger H. (2001) Preferential suppression of CXCR4-specific strains of HIV-1 by antiviral therapy. *J Clin Invest*, 107:431-438.

Fang G, Burger H, Chappey C, Rowland-Jones S, Visosky A, Chen C, Moran T, Townsend L, Murray M, and Weiser B. (2001) Analysis of transition from long-term non-progressive to progressive infection identifies sequences which may attenuate HIV-1, *AIDS Res and Hum Retroviruses*, 17:1395-1404.

Fang G, Weiser B, Visosky A, Moran T, Burger H. (2002) PCR-mediated recombination: a general method applied to construct chimeric molecular clones. PCR Cloning Protocols (second edition), B Chen and H Janes, eds., Humana Press, Totowa, NJ, 197-205.

Anastos K. Barron Y. Greenblatt R. Hessol N. Weiser B. Young M. Augenbraun M. Cohen M. Levine A. Munoz M. et al. (2002) Risk of disease progression and death in HIV infected women initiating highly active antiretroviral therapy at different stages of disease. *Arch Intern Med*,162:1973-1980.

Chakraborty R, Gillespie GM, Reinis M, Rostron T, Dong T, Philpott S, Burger H, Weiser B, Peto T, and Rowland-Jones SL. (2002) HIV-1-specific CD8 T cell responses in a pediatric slow progressor infected as a premature neonate. *AIDS*,16:2085-7.

Philpott S, Weiser B, Tarwater P, Vermund S, Kleeberger C, Gange S, Anastos K, Cohen M, Greenblatt R, Kovacs A, Minkoff H, Young M, Miotti P, Dupuis M, Chen C, and Burger H. (2003) CCR5 genotype and susceptibility to transmission of HIV-1 in women. *J Infect Dis* 187:569-575.

Landay A, Benning L, Bremer J, Weiser B, Burger H, Nowicki M, and Kovacs A. (2003) Correlates of immune activation marker changes in HIV infected and high risk HIV uninfected women who use illicit drugs. *J Infect Dis* 188:209-218.

Kemal KS, Foley B, Burger H, Anastos K, Minkoff H, Kitchen C, Philpott S, Gao W, Robison E, Holman S, Dehner C, Beck S, Meyer W, Landay A, Kovacs A, Bremer J, Weiser B. (2003) HIV-1 in genital tract and plasma of women: compartmentalization of viral sequences, coreceptor usage, and glycosylation. *Proc Natl Acad Sci* USA 100:12972-12977.

Fang G, Weiser B, Kuiken C, Philpott S, Rowland-Jones S, Plummer F, Kimani J, Shi B, Kaul R, Bwayo J, Anzala O, and Burger H. (2004) Recombination following superinfection by HIV-1. *AIDS;* 18:153-159.

Fang G, Kuiken C, Weiser B, Rowland-Jones S, Plummer F, Chen CH, Kaul R, Anzala AO, Bwayo J, Kimani J, Philpott SM, Kitchen C, Sinsheimer J, Gaschen B, Lang D, Shi B, Kemal KS, Rostron T, Brunner C, Beddows S, Sattenau Q, Paxinos E, Oyugi J, Burger H. (2004) Long-term survivors in Nairobi: complete HIV-1 RNA sequences and immunogenetic associations. *J Infect Dis,* 190:697-701.

Philpott S, Burger H, Tarwater PM, Lu M, Gange SJ, Anastos K, Cohen M, Greenblatt RM, Kovacs A, Minkoff H, Young M, Miotti P, Dupuis M, Weiser B. (2004) CCR2 Genotype and disease progression in a treated population of HIV-1 infected women. *Clin Infect Dis*, 39:861-865.

Shi B, Philpott SM, Weiser B, Kuiken C, Brunner C, Fang G, Fowke KR, Plummer FA, Rowland-Jones S, Bwayo J, Anzala AO, Kimani J, Kaul R, Burger H. (2004) Construction of an infectious HIV-1 molecular clone from an African patient with a subtype D/C recombinant virus. *AIDS Res and Hum Retroviruses*, 9: 1015-1018

Kitchen CMR, Philpott S, Burger H, Weiser B, Anastos K, Suchard MA. (2004) Evolution of HIV-1 coreceptor usage during antiretroviral therapy: a Bayesian approach, *J Virol*, 78: 11296-302.

Nag P, Kim J, Sapiega V, Landay AL, Bremer JW, Mestecky J, Reichelderfer P, Kovacs A, Cohn J, Weiser B, Baum L. (2004) Women with cervicovaginal antibody-dependent cell-mediated cytotoxicity have lower genital HIV-1 RNA loads, *J Infect Dis,* 190:1970-1978.

Philpott S, Burger H, Tsoukas C, Foley B, Anastos K, Kitchen C, Weiser B. (2005) Human immunodeficiency virus type 1 genomic RNA sequences in the female genital tract and blood: compartmentalization and intrapatient recombination. *J Virol*, 79:353-63.

Schroeder T, Burger H, Weiser B, Bengualid V, Kimani J, Anzala AO, Parker M, Lamson D, Philpott S. (2005) Characterization of intersubtype recombinant HIV-1 genomes using a non radioactive heteroduplex tracking assay. *AIDS Res and Hum Retroviruses*; 21:314-318.

Chakraborty R, Reinis M, Rostron T, Philpott S, Dong T, D'Agostino A., Musoke R, deSilva E, Stumpf M, Weiser B, Burger H, Rowland-Jones S. (2005) Nef gene sequence variation among HIV-1-infected African children. *HIV Medicine* , in press

Patent

US Patent: "Analysis of HIV-1 Co-Receptor Utilization in the Clinical Care of HIV-1-Infected Patients," patent number US 09/963,064," 2003

Present National Committee Membership

NIH Women's Interagency HIV Study (WIHS) Laboratory Committee, 1993-present (Chair of Subcommittees on Virology, 1994-98, and HIV Infection in Drug-Using Women, 1995-98)
WIHS Scientific Advisory Board, 2002-present
Chair, WIHS Scientific Working Group on HIV Virology, 2002-present

Present Local Committee Membership

Wadsworth Center Biosafety Committee, 1995 - present

Past Committee Membership

National Institutes of Health Committees
AIDS Clinical Trial Group (ACTG) Basic Research Committee, 1987-90
ACTG Virology Committee, 1987-90
AIDS Related Research Ad Hoc Study Sections, 1989-91 and 95-96
NIAID AIDS Research Review Committee, Basic Science I, 1991-1994
WIHS Review Team, 1995-1996
WIHS Executive Committee, 1997-2002

Institutional Committees
SUNY Stony Brook Biomedical Research Support Committee, 1988-91
SUNY Stony Brook Biotechnology Center Committee, 1988-91
Wadsworth Center Tuberculosis Committee, 1992
Wadsworth Center Axelrod Institute Space Allocation Committee, 1993
Wadsworth Center Axelrod Symposium Committee, 1994
Wadsworth Center Peer Review Board, 1995-1998
Chairperson, Albany Medical Center Search Committee for the Chief of the Division of HIV Medicine, 1999-2000

Membership in Professional Societies

American Society for Microbiology
American Association for the Advancement of Science
Infectious Diseases Society of America
HIV Medicine Association
International AIDS Society
New York Society of Infectious Diseases

Conference Organizer

National Conferences on Women and HIV, Los Angeles, 1997 and 1999
Satellite Meeting on HIV Infection in Women, 4th Conference on Retroviruses and Opportunistic Infections, Washington, DC, 1997

Ad Hoc Reviewer

Virology
AIDS
Journal of Virology
Clinical Infectious Diseases
Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Arlene RAMSINGH *et al.*

Serial No.: 09/879,572

Filing Date: June 12, 2001

For: COXSACKIEVIRUS B4 EXPRESSION VECTORS AND USES THEREOF

Examiner: Stacy Chen

Group Art Unit: 1648

Atty Dkt: 29025.0001

Customer No.

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PATENT TRADEMARK OFFICE

DECLARATION OF GEORGE F. VANDE WOUDE, PH.D. PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, the undersigned, declare as follows:

1. I am an expert in the field of virology, molecular biology and particularly molecular oncology. My work over the past 30+ years has focused on oncogene research. I have worked with various families of viruses and began my post-graduate career studying foot-and-mouth disease virus (FMDV), a member of the picornavirus family just like coxsackieviruses. I am an author/co-author of about 300 peer-reviewed publications, review articles and book chapters in my field. I have been the Director of the Van Andel Research Institute in Grand Rapids, MI, since 1999. Before that I was the Director of the Division of Basic Sciences of the National Cancer Institute (NCI), NIH, Bethesda, MD and Head of the Molecular Oncology Section, at the NCI-Frederick Cancer Research and Development Center in Frederick, MD (the latter position dating back to 1983. I was a section head and laboratory head at the NCI in Bethesda starting in 1972. I was elected to the National Academy of Sciences in 1993 and have been the recipient of numerous awards and honors. I have been and continue to be a member of numerous scientific review committees and advisory boards of research institutions around the world. My *Curriculum Vitae* is attached. I am

an inventor/co-inventor of a seven U.S. Patents, various corresponding foreign patents and numerous pending U.S. and foreign patent applications.

2. I was asked to reviewed relevant sections of the patent application identified above, the outstanding Office Action and the rejected claims due to my expertise in molecular genetics, virology and oncology,. My comments are based on my knowledge of the genetics of RNA viruses, DNA viruses, and mammalian cells. I am familiar with coxsackieviruses, and I spent several years working on a related virus, FMDV.

3. The claims I reviewed concern recombinant attenuated coxsackievirus B4 (CB4) virions engineered to express a foreign (or heterologous) sequence. I say "foreign" because the sequence is, or can be, derived from a different source, not a coxsackievirus of the same serotype or strain, but, for example, a truly **different** virus (such as HIV). The application also describes the use of nucleotide sequences from a variety of non-viral pathogens that encode various bacterial, parasitic and cellular, including mammalian, proteins. The CB4 virions described in the claims are genetically modified with an inserted foreign (heterologous) nucleic acid sequence that encodes a foreign (heterologous) polypeptide or peptide that can be expressed as a viral capsid fusion of the CB4 protein VP1 or as an upstream amino-terminal fusion of the CB4 protein VP4 . A major emphasis of the invention is the use of these recombinant attenuated CB4 virions as "vaccine vectors" to induce an immune response to the foreign peptide antigen engineered into CB4 virions.

4. The Patent Office's analysis of the paper by Caggana *et al. J Virol. 67*:4797-803 (1993), which was used to reject the claims on the grounds that the claims were anticipated by this paper, is incorrect as I interpret their argument. The rejection asserts that the two viral variants discussed in the paper, CB4-V and CB4-P, are "heterologous." This interpretation is improper because viral variants such as the above two are not foreign or heterologous. Genetically they are two alternative sequences of the same gene -- "alleles" or "allelic variants." I know of no definition of "foreign" or "heterologous," including the definition appearing in the application, that would result in the CB4-P and CB4-V nucleic acid sequences (or their protein products or the viruses which carry them) being called foreign or heterologous to any CB4 virus.

5. As I understand it, the Patent Office has said that it considers the sequences in Caggana *et al.* to be heterologous because "the P1 region of CB4-V was not naturally present in the genome of the CB4-P virus." Whatever this statement means, these two variants cannot be viewed as different viruses. The Patent Offices states that the Caggana reference describes a "heterologous nucleic acid **inserted** into an open reading frame that results in fusion to the capsid protein of the virion." This is backwards: the CB4-P and V sequences are "**homologous,**" the converse of "**heterologous**". Stated correctly, a portion of the nucleic acid of a CB4 virus "replaces" a *homologous* portion of a nucleic acid of the same length to yield the other variant. "Insertion" of a sequence requires the addition of coding capacity - not present in Caggana, but characteristic of the present invention where foreign sequences are inserted so that a CB4 virion expresses *de novo* a foreign peptide such as an ovalbumin peptide or various HIV peptides.

6. For the reasons cited above, I respectfully disagree with the Patent Office's conclusion that Caggana *et al.* describes "a CB4 virion with *heterologous* nucleic acid *inserted* into an open reading frame that results in fusion to the capsid protein of the virion". That is not an accurate characterization of what is described by this reference.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

May 26, 2005

Date

George F. Vande Woude

GEORGE F. VANDE WOUDE, Ph.D.
Director, Van Andel Research Institute

EDUCATION

Ph.D., Rutgers University, 1964
M.S., Rutgers University, 1962
B.A., Hofstra University, 1959

PROFESSIONAL BACKGROUND

1999-present	Director, Van Andel Research Institute, Grand Rapids, MI
1998 - 1999	Director, Division of Basic Sciences, National Cancer Institute, NIH, Bethesda, MD
1995 - 1998	Scientific Advisor to the Director for Basic Sciences, National Cancer Institute, NIH, Bethesda, MD
1983 - 1998	Director, ABL-Basic Research Program; Head, Molecular Oncology Section, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD
1981 - 1983	Chief, Laboratory of Molecular Oncology, National Cancer Institute, NIH, Bethesda, MD
1972 - 1981	Head, Human Tumor Studies Section, Viral Biology Branch, National Cancer Institute, NIH, Bethesda, MD
1965 - 1972	Research Chemist, U.S. Department of Agriculture, Plum Island Animal Disease Laboratory, Greenport, NY
1964 - 1965	Postdoctoral Research Associate, U.S. Department of Agriculture, Plum Island Animal Disease Laboratory, Greenport, NY
1960 - 1964	Research Assistant, Rutgers University, New Brunswick, NJ

HONORS, AWARDS, PROFESSIONAL RECOGNITION

U.S. Public Health Service Predoctoral Trainee, Rutgers University, 1960-1964
U.S. Department of Agriculture Postdoctoral Research Associateship Plum Island Animal Disease Laboratory, 1964-1965
Recipient, NIH Merit Award, 1982
Recipient, 1989 Pasarow Foundation Award for Cancer Research
Hassel Foundation 1990 George Khoury Memorial Lecture, Philadelphia, PA
Recipient, 1992 Lifetime Achievement Award in Technology Transfer, NASA
Honorary Doctorate of Science, Michigan State University, 1999
Grand Rapids Magazine Medical Hall of Fame Inductee, 2001
Member, National Academy of Sciences, 1993-present
Section Chair, Medical Genetics, Hematology & Oncology, National Academy of Sciences, 2004-present
Fellow, American Academy of Microbiology, 1997-present
Member, American Association for Cancer Research, 1984-present
Member, Board of Directors, American Association of Cancer Institutes, 2003-present
Member, Advisory Council, General Motors Cancer Research Foundation, 1994-present
President and CEO, Core Technology Alliance Corporation, 2004-Present

Collaborating Partner, National Dialogue on Cancer, 2000-present

Executive Board Member, Grand Rapids Clinical Oncology Program, Grand Rapids, MI, 1999-present

Alice Hogge and Arthur A. Baer Professorship and Visiting Professor, Department of Radiation and Cellular Oncology, University of Chicago, 2002-2004

Adjunct Professor, Johns Hopkins School of Medicine, Baltimore, 1985-1997

Member, Board of Directors, American Assoc for Cancer Research, 2001-2004

Member, American Association for Cancer Research's Science Policy and Legislative Affairs Committee, 2003

Kovalenko Medal Award Committee, American Association for Cancer Research, 2003

Co-Chair, Laboratory Research Awards Committee, American Association for Cancer Research, 2003

Rhoads Memorial Award Committee, American Association for Cancer Research, 2000-2001

Visiting Scientific Advisory Committee, Columbia University Comprehensive Cancer Center, New York, NY, 1986-2004

Board of Scientific Advisors, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA, 1995-present

External Advisory Committee, Karmanos Cancer Institute & Prentis Comprehensive Cancer Center at Wayne State University, Detroit, MI, 2001-present

Scientific Advisory Panel, North Shore-Long Island Jewish Research Institute, Manhasset, NY, 2002-present

Committee of Scientific Advisors, U.S. Military Cancer Institute, Washington, DC, 2001- present

Chair, External Advisory Board, Children's Cancer Research Institute, University of Texas Health Science Center, San Antonio, TX, 2003-present

Member, Governing Board of Directors, Biosciences Research & Commercialization Center, Western Michigan University, Kalamazoo, 2004-present

Advisory Board, Natl Cancer Legislation Advisory Committee/C-Change, 1999-2001 Advisory Board, Innovation Center, Michigan Life Sciences Corridor, 2000-2001

Awards Assembly, General Motors Cancer Research Foundation, 1990 -1994

National Academy of Sciences Research Briefing Panel on Oncogenes, 1984

Scientific Advisory Board of the Leonard and Madlyn Abramson Family Cancer Research Institute, University of Pennsylvania Cancer Center, 1999-2004

Board of Scientific Consultants, Memorial Sloan-Kettering Cancer Center, 1990-2002

External Advisory Committee, University of Wisconsin Comprehensive Cancer Center, Madison, WI, 1999-2002

Research Advisory Board of the Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, 1991-2000

Board of Scientific Advisors, Univ of Pennsylvania Cancer Center, 1984-1999

Scientific Advisory Committee, Vincent T. Lombardi Cancer Research Center, Georgetown University, Washington, DC, 1988-1999

Board of Scientific Counselors, Division of Cancer Etiology, National Cancer Institute, NIH, Bethesda, MD, 1985-1989

Int'l Advisory Committee, Maimonides Conferences on Cancer Research,1985-1988

Scientific Advisory Committee and Board of Managers of the Wistar Institute, Philadelphia, PA, 1985-1991

Member, Scientific Advisory Board, St. Jude Children's Hospital, 1986-1995

Scientific Advisory Board, Israel Cancer Research Fund, 1986-1990

Chairman, Board of Scientific Advisors, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA, 1987-1990

Leukemia Society of America Grant Review Subcommittee, 1987-1991

External Advisory Committee, Utah Reg Cancer Center, Salt Lake City, 1988-1994

International Review Panel of the Medical Research Council, Ottawa, Canada, 1996

EDITORIAL BOARDS / CONFERENCE ORGANIZATION

Editorial Board, *DNA and Cell Biology*, 1984-present
Co-editor (with George Klein), *Advances in Cancer Research*, 1987-present
Editorial Board, *Oncogene*, 1987-present
Member, Editorial Board, *Molecular Imaging*, 2001-present
Member, Editorial Board, *Cell Cycle*, 2001-present
Member, Editorial Board, *Molecular Cancer Research*, 2003-present
Member, Editorial Board, *Cancer Genomics and Proteomics*, 2003-present
Guest Editorial Member, *Japanese Journal of Cancer Research*, 1987-2004
Member, Editorial Board, *Cancer Letters*, 2000-2004
Research Advisory Panel, *Advances in Oncology*, 1994-2001
Associate Editor, *Cancer Research*, 1997-2000
Editor, *Journal of Virology*, 1980-1990
Associate Editor, *Cell*, 1984-1990
Founding Editor and Editor-in-Chief, *Cell Growth and Differentiation*, 1989-1996
Editorial Board, *Leukemia*, 1993-1996
Founder and President, Foundation for Advanced Cancer Studies, 1984-present
Organizer and Sponsor, Annual Oncogene Meetings, Frederick, MD, 1985-2004

PROFESSIONAL SOCIETY MEMBERSHIPS

Sigma Xi
American Chemical Society
American Society for Microbiology
American Association for Advancement of Science
American Association for Cancer Research
American Society for Cell Biology
American Academy of Microbiology

LIST OF PUBLICATIONS

1. Vande Woude GF: A new twist to DNA. Chem and Eng News, July 1961 (Letter to the Editor).
2. Vande Woude GF and Davis FF: Barrier electrophoresis: A new electrophoretic technique. Anal Biochem 6:240-250, 1963.
3. Vande Woude GF and Davis FF: Fractionation of histones in polyacrylamide gels. Anal Biochem 12:444-451, 1965.
4. Arlinghaus R, Polatnick J, and Vande Woude GF: Studies on foot-and-mouth disease virus ribonucleic acid synthesis. Virology 30:541-550, 1966.
5. Vande Woude GF, Trautman R, and Bachrach HL: The influence of the host cell, ionic strength and chymotrypsin of foot-and-mouth disease virus electrophoretic mobility. Arch Ges Virusforsch 20:71-80, 1967.
6. Vande Woude GF: Inactivity of foot-and-mouth disease virus at ionic strength dependent isoelectric points. Virology 31:436-441, 1967.
7. Vande Woude GF, Arlinghaus RB, and Polatnick J: Inhibition of ribonucleic acid methylation in the foot-and-mouth disease virus host cell. Biochem Biophys Res Commun 29:483-489, 1967.
8. Vande Woude, GF and Bachrach HL: Evidence for a single structure polypeptide in foot-and-mouth disease. Arch ges Virusforsch 23:353-356, 1968.
9. Polatnick J, Vande Woude GF, and Arlinghaus RB: Changes in protein and nucleic acid metabolism in baby hamster kidney cells infected with foot-and-mouth disease virus. Arch ges Virusforsch 23:218-226, 1968.
10. Bachrach HL and Vande Woude GF: Amino acid composition and C-terminal sequence of foot-and-mouth disease virus protein. Virology 34:282-289, 1968.
11. Ascione R and Vande Woude GF: Inhibition of host cell ribosomal ribonucleic acid methylation by foot-and-mouth disease virus. J Virol 4:727-737, 1969.
12. Vande Woude GF: Biochemical investigation of foot-and-mouth disease virus. International Pilot Conference of Foot-and-Mouth Disease Virus, New York, NY, 1969.
13. Vande Woude GF, Polatnick J, and Ascione R: Foot-and-mouth disease virus- induced alterations of baby hamster kidney cell macromolecular biosynthesis: Inhibition of ribonucleic acid synthesis. J Virol 5:458-463, 1970.
14. Vande Woude GF and Bachrach HL: The number and molecular weight of foot- and-mouth disease virus capsid proteins and the effects of maleylation. J Virol 7:250-259, 1971.
15. Ascione R and Vande Woude GF: Ribosomal factors effecting the stimulation of cell-free protein synthesis in the presence of foot-and-mouth disease virus ribonucleic acid. Biochem Biophys Res Commun 45:14, 1971.
16. Bachrach HL, Vande Woude GF, and Swaney JB: Structure and properties of FMDV and of virus-specific components. Proceedings of the Second International Congress of Virology, Budapest, 1971.
17. Ascione R, Vande Woude GF, Bachrach HL, and Ehrenfeld, E: Protein biosynthesis in cellular and cell-free systems directed by the ribonucleic acid from foot-and-mouth disease virus. Proceedings of the Second International Congress for Virology, Budapest, 1971.
18. Ascione R, Arlinghaus RB, and Vande Woude GF: Tissue culture polyribosomal systems. In Methods in Molecular Biology, Protein Biosynthesis in Non-Bacterial Systems, Vol. 2. Al Laskin and JA Last (eds). Marcel Dekker, New York, 1972.
19. Vande Woude GF, Swaney JB, and Bachrach HL: Comparison of the structural and substructural characteristics of foot-and-mouth disease and Maus-Elberfeld viruses. Biochem Biophys Res Commun 48:1222-1229, 1972.
20. Bachrach HL, Swaney JB, and Vande Woude GF: Isolation of the structural polypeptides of foot-and-mouth disease virus and analysis of their C-terminal sequences. Virology 52:520-528, 1973.
21. Swaney JB, Vande Woude GF, and Bachrach HL: Sodium dodecyl-sulfate- dependent anomalies in gel electrophoresis: Alterations in the banding patterns of foot-and-mouth disease virus polypeptides. Anal Biochem 58:337-346, 1974.
22. Vande Woude GF and Ascione R: Translation products of foot-and-mouth disease virus-infected baby hamster kidney cells. Arch ges Virusforsch 45:259-271, 1974.
23. Andrese AP, Vande Woude GF, and Walling MJ: Ultra-structural study of herpes simplex virus infection of mouse cells chronically producing Rauscher leukemia virus. J Natl Cancer Inst 53:1169-1173, 1974.
24. Holder WG, Robery WG, and Vande Woude GF: Activation of a C-type virus from the human carcinoma cell line HBT-3 by iododeoxyuridine and testosterone. Nature 249:759-762, 1974.

25. Smida J, Smidova V, Andrese A, and Vande Woude GF: Search for oncogenicity of type C particles released from mammalian cell lines transformed by avian oncornaviruses. Neoplasma 21:609-618, 1974.
26. Ascione R, Smida J, Robey WG, and Vande Woude GF: A cell-free mammalian protein-synthesizing system stimulated by RNA from avian myeloblastosis virus. Biochim Biophys Acta 395:509-524, 1974.
27. Oskarsson MK, Robey WG, Harris CL, Fischinger PJ, Haapala DK, and Vande Woude GF: A P60 polypeptide in the feline leukemia virus pseudotype of Moloney sarcoma virus with murine leukemia virus p30 antigenic determinants. Proc Natl Acad Sci USA 72:2380-2384, 1975.
28. Simonds JA, Robey WG, Graham BJ, Oie H, and Vande Woude GF: Purification of herpes virus saimiri and properties of the viral DNA. Archives of Virology 49:249-259, 1975.
29. Robey LWG, Graham BJ, Harris CL, Madden MJ, Pearson GR, and Vande Woude GF: Persistent herpes simplex virus infections established in two Burkitt lymphoma derived cell lines. J Gen Virol 32:51-62, 1976.
30. Vande Woude GF, Robey WG, Oskarsson MK, Haapala DK, Fischinger PJ, Naso RB, and Arlinghaus RB: Properties of Moloney sarcoma virus-specific p60 and its detection in transformed cells. Bibl Haemat (Basel) 43:125-127, 1976.
31. Oskarsson MK, Robey WG, Harris CL, Fischinger PJ, Haapala DK, and Vande Woude GF: A P60 polypeptide in the feline leukemia virus pseudotype of Moloney sarcoma virus with murine leukemia virus p30 antigenic determinants. RL Clark, RW Cumley and JE McCay (eds). The Year Book Medical Publishers, Inc., Haemat. (Basel) 43:125-127, 1976.
32. Robey WG, Oskarsson MK, Vande Woude GF, Naso RB, Arlinghaus RB, Haapala DK, and Fischinger PJ: Cell transformed by certain strains of Moloney sarcoma virus contain murine P60. Cell 10:79-89, 1977.
33. Oskarsson MK, Long CW, Tobe WG, Scherer M, and Vande Woude GF: Phos- phorylation and nucleic acid binding properties of ml Moloney murine sarcoma virus specific P60. J Virol 23:196-204, 1977.
34. Graham BJ, Bengali Z, and Vande Woude GF: Physical map of the origin of defective DNA in herpes simplex virus type 1 DNA. J Virol 25:878-887, 1978.
35. Oskarsson MK, Elder JH, Gautsch JW, Lerner RA, and Vande Woude GF: Chemical determination of the ml Moloney sarcoma virus $pP60^{gag}$ gene order: Evidence for unique peptides in the carboxy terminus of the polyprotein. Proc Natl Acad Sci USA 75:4694-4698, 1978.
36. Yonuschot G, Robey WG, Mushrush GW, and Vande Woude GF: Measurement of binding of terbium to DNA. Bioinorgan Chem 8:397-404, 1978.
37. Yonuschot G, Helman D, Mushrush GW, Vande Woude GF, and Robey WG: Terbium as a new solid state probe for RNA. Bioinorg Chem 8:405-418, 1978.
38. Enquist LW, Madden MJ, Schiop-Stansly P, and Vande Woude GF: Cloning of Herpes simplex type 1 DNA fragments in bacteriophage lambda vector. Science 203:541-544, 1979.
39. Vande Woude GF, Oskarsson M, Enquist LW, Nomura S, Sullivan M, and Fischinger PJ: Cloning of integrated Moloney sarcoma proviral DNA sequences in bacteriophage λ. Proc Natl Acad Sci USA 76:4464-4468, 1979.
40. Enquist LW, Vande Woude GF, Wagner M, Smiley JR, and Summers WD: Construction and characterization of a recombinant plasmid encoding the gene for the thymidine kinase of Herpes simplex type 1 virus. Gene 7:335-342, 1979.
41. Blair DG, McClements WL, Oskarsson, M, Vande Woude GF, and Fischinger P: Recombinant DNA cloning of murine oncornaviruses: Role of defined sarcoma and leukemia genetic sequences in transformation. Proceedings of the IXth International Symposium on Comparative Research in Leukemia and Related Diseases, Elsevier, NY, pp. 137-138, 1979.
42. Vande Woude GF, Oskarsson M, McClements WL, Enquist LW, Blair DG, Fischinger PJ, Maizel J, and Sullivan M: Characterization of integrated Moloney sarcoma proviruses and flanking host sequences cloned in bacteriophage λ. Cold Spring Harbor Symposium on Quantitative Biology 44:735-745, 1980.
43. Sherr CJ, Fedele LA, Oskarsson M, Sullivan M, and Vande Woude GF: Comparative structures of unintegrated linear DNA intermediates of Snyder-Theilen feline leukemia and sarcoma viruses cloned in bacteriophage lambda λ. Cold Spring Harbor Conference on Cell Proliferation, Vol. 7, pp. 613-622, 1980.
44. Oskarsson M, McClements W, Blair DG, Maizel JV, and Vande Woude GF: Properties of a normal mouse cell DNA sequence (sarc) homologous to the src sequence of Moloney sarcoma virus ("Mos"). Science 207:1222-1224, 1980.
45. Sherr CJ, Fedele LA, Oskarsson M, Maizel J, and Vande Woude GF: Molecular cloning of Snyder-Theilen feline leukemia and sarcoma viruses: Comparative studies of feline sarcoma virus with its natural helper virus and with Moloney murine sarcoma virus. J Virol 34:200-212, 1980.
46. Boyd AL, Enquist L, Vande Woude GF, and Hampar B: Activation of mouse retrovirus using herpes

simplex virus type 1 cloned DNA fragments. Virology 103:228-231, 1980.

47. Blair DG, McClements W, Oskarsson M, Fischinger P, and Vande Woude GF: Biological activity of cloned Moloney sarcoma virus DNA: terminally redundant sequences may enhance transformation efficiency. Proc Natl Acad Sci USA 77:3504-3508, 1980.
48. Dhar R, McClements W, Enquist L, and Vande Woude GF: Nucleotide sequences of integrated Moloney sarcoma provirus: long terminal repeats and their host and viral junctions. Proc Natl Acad Sci USA 77:3937-3941, 1981.
49. McClements WL, Blair DG, Oskarsson M, and Vande Woude GF: Two regions of the Moloney leukemia virus genome are required for efficient transformation by src/sarc. In Animal Virus Genetics, pp. 455-460, Proceedings of the ICN-UCLA Symposia on Molecular and Cellular Biology. B Fields, R Jaenisch and C Fox (eds). Academic Press, New York, 1980.
50. McClements WL, Enquist LW, Oskarsson M, Sullivan M, and Vande Woude GF: Frequent site-specific deletion of coliphage λ murine sarcoma virus recombinants and its use in the identification of a retrovirus integration site. J Virol 35:488-497, 1980.
51. McClements WL, Dhar R, Blair G, Enquist L, Oskarsson M, and Vande Woude GF: The long terminal repeat of Moloney sarcoma provirus. Cold Spring Harbor Symposium on Quantitative Biology, Vol. 45, pp. 699-705, 1980.
52. Watson RJ, Sullivan M, and Vande Woude GF: Structures of two spliced herpes simplex virus type 1 immediate early mRNAs. J Virol 37:431-444, 1981.
53. Blair DG, Oskarsson M, McClements L, and Vande Woude GF: The long terminal repeat of Moloney sarcoma provirus enhances transformation. In Haematology and Blood Transfusion, Modern Trends in Human Leukemia IV, Vol. 26. R Neth, RC Gallo, T Graaf, K Mannweiler and K Winkler (eds). Springer-Verlag, Berlin, pp. 400-466, 1981.
54. McClements WL and Vande Woude GF: Cloning retroviruses: Retrovirus cloning? Genetic Engineering 3:89-107, 1981.
55. Blair DG, Oskarsson MK, Wood TG, McClements WL, Fischinger PJ, and Vande Woude GF: Activation of the transforming potential of a normal cell sequence: A molecular model for oncogenesis. Science 212:941-943, 1981.
56. Denniston KJ, Madden MJ, Enquist LW, and Vande Woude GF: Characterization of coliphage lambda hybrids carrying DNA fragments from Herpes simplex virus type 1 defective interfering particles. Gene 15:365-378, 1981.
57. Straus, SE, Aulakh HS, Ruyechan WT, Hay J, Casey TA, Vande Woude GF, Owens J, and Smith HA: Structure of Varicella-Zoster virus DNA. J Virol 40:516-525, 1981.
58. Levinson, B, Khoury G, Vande Woude GF, and Gruss P: Activation of the SV40 genome by the 72 base-pair tandem repeats of Moloney sarcoma virus. Nature 295:568-572, 1982.
59. Watson R, Oskarsson M, and Vande Woude GF: Human DNA sequence homologous to the transforming gene (mos) of Moloney murine sarcoma virus. Proc Natl Acad Sci USA 79:4078-4082, 1982.
60. Swan DM, Oskarsson M, Keithley D, Ruddle FH, D'Eustachio P, and Vande Woude GF: Chromosomal localization of the Moloney sarcoma virus mouse cellular (c-mos) sequence. J Virol 44:752-754, 1982.
61. Straus SE, Owens J, Ruyechan WT, Takiff HE, Casey TA, Vande Woude GF, and Hay J: Molecular cloning and physical mapping of varicella-zoster virus DNA. Proc Natl Acad Sci USA 79:993-997, 1982.
62. Blair DG, McClements WL, and Vande Woude GF: Use of retroviral sequences in cotransfection to activate and rescue an onc gene. Proceedings of the Cold Spring Harbor Vector Meeting, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 153-157, 1982.
63. Watson RJ and Vande Woude GF: DNA sequences of an immediate-early gene (IE mRNA-5) of herpes simplex virus type I: Nucleic Acids Res 10:979-991, 1982.
64. Blair DG, Cooper CS, Oskarsson MK, Eader LA, and Vande Woude GF: New method for detecting cellular transforming genes. Science 218:1122-1125, 1982.
65. Robinson HL and Vande Woude GF: The genetic basis of retroviral-induced transformation. In Current Topics in Microbiology and Immunology, Vol. 98. W Henle, PH Hofschneider, H Koprowski, F Melchers, R Rott, HG Schweiger and PK Vogt (eds). Springer-Verlag, Heidelberg, pp. 11-16, 1982.

66. Blair DG and Vande Woude GF: Moloney sarcoma virus: A model for trans- formation by endogenous cellular genes. In Advances in Viral Oncology, Vol. 1., G Klein (ed.). Raven Press, New York, pp. 189-205, 1982.
67. McGeady ML, Jhappan R, Ascione R, and Vande Woude GF: In vitro methylation of specific regions of the cloned moloney sarcoma virus genome inhibits its transforming activity. Mol Cell Biol 3:305-314, 1983.
68. Wood T, McGeady ML, Blair DG, and Vande Woude GF: Long terminal repeat enhancement of v-mos transforming activity: Identification of essential regions. J Virol 46:726-736, 1983.
69. Blair DG, Cooper CS, Oskarsson MK, Eader LA, and Vande Woude GF: Tumori- genesis by transfected cells in nude mice: A new method for detecting cellular transforming genes. In Oncogenes and Retroviruses: Evaluation of Basic Findings and Clinical Potential, pp. 79-90. Alan R. Liss, Inc., New York, 1983.
70. Woodworth A, Oskarsson M, Blair DG, McGeady ML, Tainsky M, and Vande Woude GF: Biological properties of the human DNA sequence homologous to the mos transforming gene of Moloney sarcoma virus. In Genes and Proteins in Oncogenesis, pp. 233-240. IB Weinstein and HJ Vogel (eds). Academic Press, New York, 1983.
71. Wood T, Blair D, McGeady ML, and Vande Woude G: Sequences involved in the activation of the transforming potential of a normal cellular gene, c-mos. In Enhancers and Eukaryotic Gene Expression, pp. 110-117. Y Gluzman and T Shenk (eds). Cold Spring Harbor Laboratory, New York, 1983.
72. Wood TG, Blair DG, and Vande Woude GF: Moloney sarcoma virus: analysis of RNA and DNA structure in cells transformed by subgenomic proviral DNA fragments. In Perspectives on Genes and the Molecular Biology of Cancer, pp. 299-306. DL Robberson and GF Saunders (eds). Raven Press, New York, 1983.
73. Blair DG, Wood TG, Woodworth AM, McGeady ML, Oskarsson MK, Propst F, Tainsky M, Cooper CS, Watson R, Baroudy BM, and Vande Woude GF: Properties of the mouse and human mos oncogene loci. In Cancer Cells: Oncogenes and Viral Genes, Vol. 2. GF Vande Woude, AJ Levine, WC Topp and JD Watson (eds). Cold Spring Harbor Laboratory, New York, pp. 281-289, 1984.
74. Cooper CS, Blair DG, Oskarsson MK, Tainsky MA, Eader LA, and Vande Woude GF: Characterization of human transforming genes from chemically transformed, teratocarcinoma and pancreatic carcinoma cell lines. Cancer Res 44:1-10, 1984.
75. Tainsky MA, Cooper CS, Giovanella BC, and Vande Woude GF: An activated ras^N gene is detected in late but not early passage human PA1 teratocarcinoma cells. Science 225:643-645, 1984.
76. Cooper CS, Park M, Blair DG, Tainsky MA, Huebner K, Croce CM, and Vande Woude GF: Molecular cloning of a new transforming gene from a chemically-transformed human cell line. Nature 311:29-33, 1984.
77. Wood TG, McGeady ML, Baroudy B, Blair DG, and Vande Woude GF: Mouse c-mos oncogene activation is prevented by upstream sequences. Proc Natl Acad Sci USA 81:7817-7821, 1984.
78. Levine AJ, Vande Woude GF, Topp WC, and Watson JD (eds). Cancer Cells: The Transformed Phenotype, Vol. 1, Cold Spring Harbor Laboratory, New York, 1984.
79. Vande Woude GF, Levine AJ, Topp WC, and Watson JD (eds). Cancer Cells: Oncogenes and Viral Genes, Vol. 2, Cold Spring Harbor Laboratory, New York, 1984.
80. Propst F, Vande Woude GF: A novel transposon-like repeat interrupted by an LTR element occurs in a cluster of B1 repeats in the mouse c-mos locus. Nucl Acids Res 12:8381-8392, 1984.
81. Weinberg RA, Erikson R, Rapp F, Sporn M, Todaro G, Vande Woude GF, and Wigler M: Report of the Research Briefing Panel on the Biology of Oncogenes. In Research Briefings, pp. 57-67. Natl Acad Press, Washington, D.C., 1984.
82. Vande Woude GF and Gilden RV: Principles of cancer biology: The molecular biology of cancer. In Cancer: Principles & Practices of Oncology, V DeVita, S Hellman and SA Rosenberg (eds). JB Lippincott Co., Philadelphia, pp. 23-33, 1985.
83. Propst F and Vande Woude GF: Expression of c-mos proto-oncogene transcripts in mouse tissues. Nature 315:516-518, 1985.
84. Seth A and Vande Woude GF: Nucleotide sequence and the biochemical activities of the HT1MSV mos gene. J Virol 56:144-152, 1985.

85. White R, Woodward S, Leppert M, O'Connell P, Hoff M, Herbst J, Lalouel J, Dean M, and Vande Woude G: A closely linked genetic marker for cystic fibrosis. Nature 318:382-384, 1985.
86. Dean M, Park M, LeBeau M, Robins T, Diaz M, Rowley J, Blair D, and Vande Woude G: The human met oncogene is related to the tyrosine kinase oncogenes. Nature 318:385-388, 1985.
87. Vande Woude GF, Blair DG, McGeady ML, Wood T, and Propst F: Properties of the mouse mos proto-oncogene locus. Proceedings for the International Advanced Course on "Human Preleukemia", Rome, Italy, November 11-13, 1985.
88. Vande Woude GF, Dean M, Gonzatti-Haces M, Iyer A, Kaul K, Robins T, Park M, and Blair DG: Properties of the met oncogene. In Gene Amplification and Analysis: Oncogenes. Vol. 4. TS Papas and GF Vande Woude (eds). Elsevier Press, Amsterdam, pp. 239-252, 1986.
89. Robins TC, Jhappan C, Chirikjian J, and Vande Woude GF: Molecular cloning of the intronless EJ ras oncogene using a murine retrovirus shuttle vector. Gene Anal Techn 3:12-16, 1986.
90. Jhappan C, Vande Woude GF, and Robins TS: Transduction of host cellular sequences by a retroviral "shuttle" vector. J Virol 60:750-753, 1986.
91. McGeady ML, Wood TG, Maizel JV, and Vande Woude GF: Sequences upstream to the mouse c-mos oncogene may function as a transcription termination signal. DNA 5:289-298, 1986.
92. Seth A, Lapis P, Vande Woude GF, and Papas T: High-level expression vectors to synthesize unfused proteins in Escherichia coli. Gene 42:49-57, 1986.
93. Park M, Dean M, Cooper CS, Schmidt M, O'Brien SJ, Blair DG, and Vande Woude GF: Mechanism of met oncogene activation. Cell 45:895-904, 1986.
94. Blair DG, Oskarsson MK, Seth A, Dunn KJ, Dean M, Zweig M, Tainsky MA, and Vande Woude GF: Analysis of the transforming potential of the human homolog of mos. Cell 46:785-794, 1986.
95. Park M, Gonzatti M, Dean M, Blair DG, Testa JR, Bennett DD, Copeland T, Oroszlan S, and Vande Woude GF: The met oncogene: a new member of the tyrosine kinase family and a marker for cystic fibrosis. Cold Spring Harbor Symposia on Quantitative Biology 51:967-975, 1986.
96. White R, Leppert M, O'Connel P, Nakamura Y, Woodward S, Hoff M, Herbst J, Dean M, Vande Woude GF, Lathrop GM, and Lalouel J-M: Further linkage data on cystic fibrosis: The Utah Study. Am J Hum Genet 39:694-698, 1986.
97. Vande Woude GF, Oskarsson MK, McGeady ML, Seth A, Propst F, Schmidt M, Paules R, and Blair DG: Sequences which influence the transforming activity and expression of the mos oncogene. In Advances in Viral Oncology, Vol. 6, G Klein (ed.). Raven Press, pp. 71-81, 1987.
98. Vande Woude GF, Blair DG, McGeady ML, Wood T, and Propst F: Properties of the mouse mos proto-oncogene locus. Haematologica 72:2-5, 1987.
99. Gonzatti-Haces M, Park M, Dean M, Blair DG, and Vande Woude GF: The human met oncogene is a member of the tyrosine kinase family. In Oncogenes and Cancer. SA Aaronson et al. (ed.). Japan Sci Soc Press, Tokyo. pp 221-232, 1987.
100. Dean M, Park M, and Vande Woude GF: Characterization of the rearranged tpr-met oncogene breakpoint. Mol Cell Biol 7:921-924, 1987.
101. Seth A, Priel E, and Vande Woude GF: Nucleoside triphosphate-dependent nucleic- acid-binding properties of mos protein. Proc Natl Acad Sci USA 84:3560-3564, 1987.
102. Dean M, Park M, Gonzatti-Haces M, Iyer A, Copeland T, Oroszlan S, Blair DG, and Vande Woude GF: The met oncogene: From morphological transformation to cystic fibrosis. In Lectures and Symposia 14th Intl Cancer Congress, Budapest, Vol. 1, pp. 1-16, 1987.
103. Propst F, Rosenberg MP, Iyer A, Kaul K, and Vande Woude GF: c-mos proto- oncogene RNA transcripts in mouse tissues: structural features, developmental regulation and localization in specific cell types. Mol Cell Biol 7:1629-1637, 1987.
104. Collins FS, Drumm ML, Cole JL, Lockwood WK, Vande Woude GF, and Iannuzzi MC: Construction of a general human chromosome jumping library, with application to cystic fibrosis. Science 235:1046-1049, 1987.
105. Hapel AJ, Vande Woude GF, Campbell HD, Young IG, and Robins T: Generation of an autocrine leukaemia using a retroviral expression vector carrying the interleukin-3 gene. Lymphokine Res 5:249-254,

1987.

106. O'Connell M, Leppert Y, Nakamura Y, Dean M, Park M, Vande Woude GF, Farrall M, Wainwright B, Williamson R, Lathrop GM, Lalouel J-M, and White R: DNA markers for the cystic fibrosis locus. In Genetics and Epithelial Cell Dysfunction in Cystic Fibrosis. JR Riordan and M Buchwald (eds). Alan R. Liss, Inc., NY, pp. 127-137, 1987.
107. Dean M, Park M, O'Connel P, White R, and Vande Woude GF: The met oncogene: A tyrosine kinase and marker for cystic fibrosis. In Molecular Mechanisms in the Regulation of Cell Behavior. Alan R. Liss, Inc. NY, pp. 107-112, 1987.
108. Park M, Dean M, Kaul K, Braun MJ, Gonda MA, and Vande Woude GF: Sequence of met proto-oncogene cDNA has features characteristic of the tyrosine kinase family of growth factor receptors. Proc Natl Acad Sci USA 84: 6379-6383, 1987.
109. Dean M, O'Connell P, Leppert M, Park M, Amos JA, Phillips DG, White R, and Vande Woude GF: Three additional DNA polymorphisms in the met gene and the D7S8 locus: use in prenatal diagnosis of cystic fibrosis. J Pediatrics 111:490-495, 1987.
110. Dean M, Kozak C, Robbins J, Callahan R, O'Brien S, and Vande Woude GF: Chromosomal localization of the met proto-oncogene in the mouse and cat genome. Genomics 1:167-173, 1987.
111. Tainsky MA, Shamanski FL, Blair D, and Vande Woude G: Human recipient cell for oncogene transfection studies. Mol and Cell Biol 7:1280-1284, 1987.
112. McGeady ML, Wood TG, Blair DG, Seth A, Propst F, Oskarsson M, Schmidt M, and Vande Woude GF: CIS regulatory control of mos oncogene expression. Molecular Biology and Differentiation of Cancer Cells (Oncogenes, Growth Factors, Receptors). Vol. 2. Lapis and Eckhardt (eds). Akademiai Kiado, Budapest, 1987. (Proceedings of the 14th International Cancer Congress, Budapest, Hungary, August 21-27, 1986).
113. Khillan JS, Oskarsson MK, Propst F, Kuwabara T, Vande Woude GF, and Westphal H: Genes and Development: Defects in lens fiber differentiation are linked to c-mos overexpression in transgenic mice. Genes and Devel 1:1327-1335, 1987.
114. Dean M, Park M, Kaul K, Blair D, and Vande Woude GF: Activation of the met proto-oncogene in a human cell line. Haematology and Blood Transfusion, Vol. 31. Modern Trends in Human Leukemia VII. Neth, Gallo, Greaves, Kabisch (eds). Springer-Verlag, Berlin, Heidelberg, 1987.
115. Seth A and Vande Woude GF: The mos Oncogene. In The Oncogene Handbook. EP Reddy, AM Skalka and T Curran (eds). Elsevier Science Publishers B.V., Amsterdam, The Netherlands, pp 195-211, 1988.
116. Leppert M, O'Connell P, Sears T, Park M, Dean M, Vande Woude GF, Latrop M, and White R: DNA probes in the cystic fibrosis region of chromosome 7. In Nucleic Acid Probes in Diagnosis of Human Genetic Disease, Alan R. Liss, Inc., NY, pp. 101-113, 1988.
117. Gonzatti-Haces M, Seth A, Park M, Copeland T, Oroszlan S, and Vande Woude GF: Characterization of the tpr-met oncogene p65 and the met proto-oncogene cell surface p140 tyrosine kinases. Proc Natl Acad Sci USA 85:21-25, 1988. Schmidt M, Oskarsson MK, Dunn JK, Blair DG, Hughes S, Propst F, and
118. Vande Woude GF: Chicken homolog of the mos proto-oncogene. Mol Cell Biol 8:923-929, 1988. Park M, Testa JR, Blair DG, Parsa NZ, and Vande Woude GF: Two rearranged met alleles in MNNG-HOS cells reveal the orientation of met on chromosome 7 to other markers tightly linked to the cystic fibrosis locus. Proc Natl Acad Sci USA 85:2667-2671, 1988.
119. Propst F, Rosenberg MP, Oskarsson MK, Russell LB, Nguyen-Huu MC, Nadeau J, Jenkins NA, Copeland NG, and Vande Woude GF: Genetic analysis and developmental regulation of testis-specific RNA expression of Mos, Abl, actin and Hox-1.4. Oncogene 2:227-233, 1988.
120. Keshet E, Rosenberg M, Mercer JA, Propst F, Vande Woude GF, Jenkins NA, and Copeland NG: Developmental regulation of ovarian-specific mos expression. Oncogene 2:235-240, 1988.
121. Paules RS, Propst F, Dunn KJ, Blair DG, Kaul K, Palmer AE, and Vande Woude GF: Primate c-mos proto-oncogene structure and expression: Transcription initiation both upstream and within the gene in a tissue-specific manner. Oncogene 3:59-68, 1988.
122. Testa JR, Parsa NZ, Le Beau MM, and Vande Woude GF: Localization of the proto- oncogene mos to 8q11-q12 in situ chromosomal hybridization. Genomics 3:44-47, 1988.
123. Propst F, Rosenberg MP, and Vande Woude GF: Proto-oncogene expression in germ cell development.

Trends in Genetics 4:183-187, 1988.

124. Sagata N, Oskarsson M, Copeland T, Brumbaugh J, and Vande Woude GF: Function of c-mos proto-oncogene product in meiotic maturation in Xenopus oocytes. Nature 335:519-525, 1988.
125. Park M and Vande Woude GF: Oncogenes: genes associated with neoplastic disease. In The Metabolic Basis of Inherited Disease, Vol. 1, Sixth Edition. CR Scriver, AL Beaudet, WS Sly and D Valle (eds). McGraw-Hill, Inc., New York, pp. 251-276, 1989.
126. Dean M and Vande Woude GF: Principle of molecular cell biology of cancer: Introduction to methods in molecular biology. In Cancer: Principles and Practice of Oncology, Vol. 1, Third Edition. V DeVita, S Hellman and SA Rosenberg (eds). JB Lippincott Co., Philadelphia, pp. 14-30, 1989.
127. Vande Woude S and Vande Woude GF: Principles of molecular cell biology of cancer: General aspects of gene regulation. In Cancer: Principles and Practice of Oncology, Vol. 1, Third Edition. V DeVita, S Hellman and SA Rosenberg (eds). JB Lippincott Co., Philadelphia, pp. 31-44, 1989.
128. Seth A, Konopka AK, and Vande Woude GF: Localization of nucleic acid binding domain in mos. In Advances in Gene Technology: Protein Engineering and Production. Proceedings of the 1988 Miami Bio/Technology Winter Symposium. ICSU Short Reports, Volume 8. K Brew, F Ahmad, H Bialy, S Black, RE Fenna, D Puett, WA Scott, J Van Brunt, RW Voellmy, WJ Whelan and JF Woessner (eds). IRL Press, Washington, DC, pg. 82, 1988.
129. Seth A, Blair DG, Dunn KJ, Fisher RJ, and Vande Woude GF: High-level expression of human growth hormone-mos (p58hGH-mos) product in C-127 cells. In Advances in Gene Technology: Protein Engineering and Production. Proceedings of the 1988 Miami Bio/Technology Winter Symposium. ICSU Short Reports, Volume 8. K Brew et al., (eds). IRL Press, Washington, DC, pg. 112.
130. Park M and Vande Woude GF: Principles of molecular cell biology of cancer: Oncogenes. In Cancer: Principles and Practice of Oncology, Vol. 1, Third Edition. V DeVita, S Hellman and SA Rosenberg (eds). JB Lippincott Co., Philadelphia, pp 45-66, 1989.
131. Propst F, Vande Woude GF, Jenkins NA, Copeland NG, Lee BK, Hunt PA, and Eicher EM: The mos proto-oncogene maps near the centromere on mouse chromosome 4. Genomics 5:118-123, 1989.
132. Paules RS, Buccione R, Moschel RC, Vande Woude GF, and Eppig JJ: Mouse mos protooncogene product is present and functions during oogenesis. Proc Natl Acad Sci USA 86:5395-5399, 1989.
133. Vande Woude GF, Gonzatti-Haces M, Iyer A, Park M, Testa JR, Oskarsson M, Paules RS, Propst F, and Sagata N: The mos and met oncogenes: Transformation and reverse genetics. In The Regulation of Proliferation and Differentiation in Normal and Neoplastic Cells, Academic Press, Inc., 143-164, 1989.
134. Sagata N, Daar I, Oskarsson M, Showalter S, and Vande Woude GF: The product of the mos proto-oncogene as a candidate "initiator" for oocyte maturation. Science 245:643-646, 1989.
135. Sagata N, Watanabe N, Vande Woude GF, and Ikawa Y: The c-mos proto- oncogene product is a cytostatic factor (CSF) responsible for meiotic arrest in vertebrate eggs. Nature 342:512-518, 1989.
136. Watanabe N, Vande Woude GF, Ikawa Y, and Sagata N: Specific proteolysis of the c-mos proto-oncogene product by calpain upon fertilization of Xenopus eggs. Nature 342:505-511, 1989.
137. Iyer A, Kmiecik TE, Park M, Daar I, Blair D, Dunn KJ, Sutrave P, Ihle JN, Bodescot M, and Vande Woude GF: Structure, tissue-specific expression and transforming activity of the mouse met protooncogene. Cell Growth and Diff 1:87-95, 1990.
138. Vande Woude GF, Buccione R, Daar I, Eppig JJ, Oskarsson M, Paules R, Sagata N, and Yew N: mos proto-oncogene function. In The Proceedings of the Symposium on Proto-oncogenes in Cell Development, John Wiley and Sons Limited, West Sussex, England, pp. 147-162, 1990.
139. Vande Woude GF, Buccione R, Daar I, Eppig JJ, Oskarsson M, Paules R, Sagata N, and Yew N: mos protooncogene function. In Gene Regulation, Oncogenesis and AIDS, Portfolio Publishing Company of Texas, Inc., The Woodlands, TX, pp. 49-65, 1990.
140. Schulz N and Vande Woude GF: mos proto-oncogene product and cytostatic factor. In Proceedings of the VII Ovarian Workshop entitled "Signaling Mechanisms and Gene Expression in the Ovary". G Gibori (ed.). Springer-Verlag (Pub.), New York, NY, Serono Symposia, Norwell, MA, pp. 111-128, 1991.
141. Propst F, Cork LC, Kovatch RM, and Vande Woude GF: Progressive neurodegenerative changes in transgenic mice overexpressing mos oncogenes in the brain. In Proceedings of the Fifth World Congress

of Biological Psychiatry. G Racagni, N Brunello and T Fukuda (eds.). Florence, Italy, pp. 443-445.

142. Propst F, Rosenberg MP, Cork LC, Kovatch RM, Rauch S, Westphal H, Khillan J, Schulz NT, Vande Woude GF, and Newmann PE: Neuro-pathological changes in transgenic mice carrying copies of a transcriptionally activated Mos protooncogene. Proc Natl Acad Sci USA 87:9703-9707, 1991.
143. Vande Woude GF, Zhou R, Paules RS, Schulz N, Daar I, Yew N, Sagata N, Watanabe N, and Oskarsson M: mos proto-oncogene product and cell cycle control. Proceedings of the Cold Spring Harbor Symposium on Origins of Human Cancer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 65-75, 1991.
144. Zhou R, Oskarsson M, Paules RS, Schulz N, Cleveland D, and Vande Woude GF: Ability of the c-mos product to associate with and phosphorylate tubulin. Science 251:671-675, 1991.
145. Bottaro DP, Rubin JS, Faletto DL, Chan AM-L, Kmiecik TE, Vande Woude GF, and Aaronson SA: Identification of the HGF receptor as the c-met protooncogene product. Science 251:802-804, 1991.
146. Zhou R, Shen R, Pinto da Silva P, and Vande Woude GF: In vitro and in vivo characterization of pp39mos association with tubulin. Cell Growth and Diff 2:257-265, 1991.
147. Daar I, Nebreda AR, Yew N, Sass P, Paules R, Santos E, Wigler M, and Vande Woude GF: The ras oncoprotein and M-phase activity. Science 253:74-76, 1991.
148. Daar I, Paules RS, and Vande Woude GF: A characterization of cytostatic factor activity from Xenopus eggs and c-mos-transformed cells. J Cell Biol 114:329-335, 1991.
149. Daar I, White GA, Schuh SM, Ferris DK, and Vande Woude GF: tpr-met oncogene product induces MPF activation in Xenopus oocytes. Mol Cell Biol 11:5985-5991, 1991.
150. Schulz N and Vande Woude GF: A Transgenic mouse model for Men 2. In AIDS Research and Human Retroviruses: Abstracts from the 1991 Annual Meeting sponsored by the Laboratory of Tumor Cell Biology, 1991.
151. Okazaki K, Furuno N, Watanabe N, Ikawa Y, Vande Woude GF, and Sagata N: Correlation between physiological and transforming activities of the c-mos proto-oncogene product and identification of an essential Mos domain for these activities. Jap J Cancer Res 82:250-253, 1992.
152. Daar I, Zhou R, Shen R-L, Nebreda A, Oskarsson M, Santos E, Pinto da Silva P, Masui Y, and Vande Woude GF: MOS and RAS; two oncoproteins that display M-phase activity. Cold Spring Harbor Laboratory Symposia on Quantitative Biology, Vol. 56, pp. 477-488, 1992.
153. Schulz N, Propst F, Rosenberg MP, Linnoila RI, Paules RS, Kovatch R, Ogiso Y, and Vande Woude GF: Pheochromocytomas and C-cell thyroid neoplasms in transgenic c-Mos mice: A model for the human multiple endocrine neoplasia type 2 syndrome. Cancer Res 52:450-455, 1992.
154. Faletto DL, Tsarfaty I, Kmiecik TE, Gonzatti M, Suzuki T, and Vande Woude GF: Evidence for noncovalent clusters of the c-met proto-oncogene product. Oncogene 7:1149-1157, 1992.
155. Yew N, Mellini ML, and Vande Woude GF: Meiotic initiation by the mos protein in Xenopus. Nature 355:649-652, 1992.
156. Schulz N, Propst F, Rosenberg MP, Linnoila RI, Paules RS, Schulte D, and Vande Woude GF: Patterns of neoplasia in c-mos transgenic mice and their relevance to multiple endocrine neoplasia. Henry Ford Hospital Medical Journal 40:307-311, 1992.
157. Vande Woude GF: Hepatocyte growth factor: Mitogen, motogen and morphogen. Jap J Can Res, Vol. 83, cover article, 1992.
158. Higuchi O, Mizuno K, Vande Woude GF, and Nakamura T: Expression of c-met proto-oncogene in COS cells induces the signal transducing high-affinity receptor for hepatocyte growth factor. FEBS Lett 301:282-286, 1992.
159. Zhou R, Daar IO, Ferris D, White G, Paules R, and Vande Woude GF: pp39mos is associated with p34cdc2 kinase in c-mosxe-transformed NIH/3T3 cells. Mol Cell Biol 12:3583-3589, 1992.
160. Paules RS, Resnick J, Kasenally AB, Ernst MK, Donovan P, and Vande Woude GF: Characterization of activated and normal mouse mos gene in murine 3T3 cells. Oncogene 7:2489-2498, 1992.
161. Tsarfaty I, Resau JH, Rulong S, Keydar I, Faletto DL, and Vande Woude GF: The met protooncogene receptor and lumen formation. Science 257:1258-1261, 1992.

162. Rong S, Bodescot M, Blair D, Dunn J, Nakamura T, Mizuno K, Park M, Chan A, Aaronson S, and Vande Woude GF: Tumorigenicity of the met protooncogene and the gene for hepatocyte growth factor. Mol Cell Biol 12:5152-5158, 1992.

163. Faletto DL, Kaplan DR, Halverson DO, Rosen ER, and Vande Woude GF: In Hepatocyte Growth Factor/Scatter Factor (HGF-SF) and The c-met Receptor. Signal transduction in c-met mediated motogenesis. ID Goldberg and E Rosen (eds). Birkhauser Verlag Company, pp 107-130, 1993.

164. Vande Woude GF: In Advances in Oncology. The mos oncogene and neoplasia, Vol. 8. GP Canellos and L Weiss (eds). Cligott Communications, pp. 2-9, 1992.

165. Kmiecik TE, Keller JR, and Vande Woude GF: Hepatocyte growth factor is a synergistic factor for the growth of hematopoietic progenitor cells. Blood 80:2454-2457, 1992.

166. Vande Woude S and Vande Woude GF: Principles of molecular cellular biology of cancer: Introduction to methods in molecular biology. In Cancer: Principles and Practice of Oncology, Fourth Edition. V DeVita, S Hellman and SA Rosenberg (eds). JB Lippincott Co., Philadelphia, pp. 3-22, 1993.

167. Perkins AS and Vande Woude GF: Principles of molecular cellular biology of cancer: Principles of Molecular Cell Biology of Cancer: Oncogenes. In Cancer: Principles and Practice of Oncology, Fourth Edition. V DeVita, S Hellman and SA Rosenberg (eds). JB Lippincott Co., Philadelphia, pp. 35-59, 1993.

168. Daar I, Yew N, and Vande Woude GF: Inhibition of mos-induced oocyte maturation by protein kinase A. J Cell Biol 120:1197-1202, 1993.

169. Yew N, Strobel M, and Vande Woude GF: Mos and the cell cycle: The molecular basis of the transformed phenotype. Current Opinion in Gen & Dev 3:19-25, 1993.

170. Posada J, Yew N, Ahn NG, Vande Woude GF, and Cooper JA: Mos stimulates MAP kinase in Xenopus oocytes and activates a MAP kinase kinase in vitro. Mol Cell Biol 13:2546-2553, 1993.

171. Rong S, Oskarsson M, Faletto DL, Tsarfaty I, Resau J, Nakamura T, Rosen E, Hopkins R, and Vande Woude GF: Tumorigenesis induced by co-expression of human hepatocyte growth factor and the human met protooncogene leads to high levels of expression of the ligand and receptor. Cell Growth & Diff 4:563-569, 1993.

172. Vande Woude GF, Choi T, Zhou R, Murakami M, Matten W, and Fukasawa K: Mos proto-oncogene and cell cycle regulation. In The Cell Cycle: Regulators, Targets and Clinical Applications. V.W. Hu (ed.). Plenum Press, New York, pp. 247-249, 1994.

173. Vande Woude GF: Proceedings of the Otto Herz Symposium, September 26-29, 1993, Tel Aviv, Israel.

174. Rong S, Jeffers M, Resau JH, Tsarfaty I, Oskarsson M, and Vande Woude GF: Met Expression and Sarcoma Tumorigenicity. Cancer Research 53:5355-5360, 1993.

175. Tsarfaty I, Rong S, Resau JH, Rulong S, Pinto da Silva P, and Vande Woude GF: The Met proto-oncogene Mesenchymal to Epithelial Cell Conversion. Science 263:98-101, 1994.

176. Matten W and Vande Woude GF: Xenopus laevis maturation and early embryonic cell cycles", In Seminars in Developmental Biology, (Academic Press), Vol. 5, pp. 173-181, 1994.

177. Vande Woude GF: "Mos", In The Protein Kinase Factsbook. G Hardie and S Hanks (eds). Academic Press, pp. 158-160, 1995.

178. Rong S, Segal S, Anver M, Resau JH, and Vande Woude GF: Invasiveness and metastasis of NIH/3T3 cells induced by Met-HGF/SF autocrine stimulation. Proc Natl Acad Sci 91:4731-4735, 1994.

179. Murakami MS, Strobel M, and Vande Woude GF: Cell Cycle Regulation, Oncogenes, and Antineoplastic Drugs. In The Molecular Basis of Cancer. J Mendelsohn, PM Howley, MA Israel and LA Liotta (eds). W.B. Saunders Co., Philadelphia, PA, pp. 3-17, 1994.

180. Matten W, Daar I, and Vande Woude GF: Protein kinase A acts at multiple points to inhibit Xenopus oocyte maturation. Mol Cell Biol 14:4419-4426, 1994.

181. Matten W and Vande Woude GF: Microinjection into Xenopus Oocytes. In Oncogene Techniques. PK Vogt and IM Verma (eds). Academic Press, New York, NY, Volume 254, pp. 458-466, 1995.

182. Vande Woude, GF: Embryology - On the loss of Mos. Nature 370:20-21, 1994.

183. Jung W, Castren E, Odenthal M, Vande Woude GF, Ishii T and Dienes H-P, Lindholm D, and Schirmacher P: Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor, c-met

in mammalian brain. J Cell Biol 126:485-494, 1994.

184. Fukasawa K, Murakami MS, Blair DG, Kuriyama R, Hunt T, Fischinger P, and Vande Woude GF: Similarities between somatic cells overexpressing mos oncogene and oocytes during meiotic interphase. Cell Growth & Diff 5:1093-1103, 1994.
185. Sheets MD, Fox CA, Hunt T, Vande Woude G, and Wickens M: The 3'- untranslated regions of c-mos and cyclin in RNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes & Devel 8:926-938, 1994.
186. Wang XM, Yew N, Peloquin JG, Vande Woude GF, and Borisy GG: Mos oncogene product associates with kinetochores in mammalian somatic cells and disrupts mitotic progression. Proc Natl Acad Sci USA 91:8329-8333, 1994.
187. Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, and Ahn NG: Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265:966-970, 1994.
188. Fukasawa K and Vande Woude GF: Mos overexpression in Swiss 3T3 cells induces meiotic-like alterations of the mitotic spindle. Proc Natl Acad Sci USA 92:3430-3434, 1995.
189. Rong S, Donehower LA, Hansen MF, Strong L, Tainsky M, Resau JH, Hudson E, Tsarfaty I, and Vande Woude GF: Met protooncogene product is overexpressed in tumors of p53 deficient mice and tumors of Li-Fraumeni patients. Cancer Res 55:1963-1970, 1995.
190. Fukasawa K, Rulong S, Resau J, Pinto da Silva P, and Vande Woude GF: Overexpression of mos oncogene product in Swiss 3T3 cells induces apoptosis preferentially during S-phase. Oncogene 10:1-8, 1995.
191. Fukasawa K, Rulong S, Resau JH, Matten W, Murakami M, Choi T, and Vande Woude GF: Different consequences of v-Mos expression in somatic cells: Transformation, growth-arrest, and apoptosis". In Proceedings of the 47th Annual Symposium on Cell Death in Development and Cancer, 48-50, 1994.
192. Rong S and Vande Woude GF: Autocrine mechanism for met proto-oncogene tumorigenicity. Cold Spring Harbor Symp on Quant Biol, Molecular Genetics of Cancer, Volume 59:629-636, 1994.
193. Fukasawa K, Zhou R, Matten WT, Armstrong AJ, Daar I, Oskarsson M, Sathyanarayana BK, Wood TG, and Vande Woude GF: Mutagenic analysis of functional domains of the mos proto-oncogene and identification of the site important for its DNA binding activity. Oncogene 11:1447-1457, 1995.
194. Resing KA, Mansour SJ, Hermann AS, Johnson RS, Candia JM, Fukasawa K, Vande Woude GF, and Ahn NG: Determination of v-mos catalyzed phosphorylation sites and autophosphorylation sites on MAP kinase kinase by ESI/MS. Biochemistry 34:2610-2620, 1995.
195. Cortner J, Vande Woude GF, and Rong S: The Met-HGF/SF autocrine signaling mechanism is involved in human sarcomagenesis. In Epithelial Mesenchymal Interactions in Cancer. I Goldberg and E Rosen (eds). Virkhauser Verlag, pp. 89-121, 1995.
196. Vande Woude GF: Conference Summary In Accomplishments in Cancer Research, General Motors Cancer Research Foundation, JG Fortner and JE Rhoads (eds) J.B. Lippincott Co., Philadelphia, 247-253, 1994.
197. Rulong S, Zhou R, Tsarfaty I, Hughes S, Vande Woude G, and Pinto da Silva, P: Immunogold labeling of oncogenic and tumor related proteins. Microscopy Res & Tech 31:159-173, 1995.
198. Jeffers M, Rong S, and Vande Woude GF: Enhanced tumorigenicity and invasion/ metastasis by HGF/SF-Met signaling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol 16:1115-1125, 1996.
199. Choi T, Rulong S, Resau J, Fukasawa K, Matten W, Kuriyama R, Mansour S, Ahn N, Vande Woude GF: A novel system for analyzing spindle formation during meiosis I. Proc Natl Acad Sci USA 93:4730-4735, 1996.
200. Fukasawa K, Choi T, Rulong S, Kuriyama R, and Vande Woude GF: Abnormal centrosome amplification and genetic instability in the absence of p53. Science 271:1744-1747,1996.
201. Cortner J and Vande Woude GF: Essentials of Molecular Biology. In Cancer: Principles and Practices of Oncology, 5th Edition. VT DeVita, S Hellman, and SA Rosenberg (eds). J.B. Lippincott Co., Philadelphia, PA, pp. 3-33, 1997.

202. Fukasawa K, Choi T and Vande Woude GF: Mos, Meiosis and Cellular Transformation. In Tumor Biology, Regulation of Cell Growth, Differentiation and Genetics in Cancer, Volume H99, AS Tsiftsoglou, AC Sartorelli, DE Housman, TM Dexter (eds). Springer-Verlag, Berlin, pp. 59-71, 1996.
203. Matten WT, Copeland TD, Ahn NG and Vande Woude GF: A positive feedback signal from MAP kinase to Mos during Xenopus oocyte maturation. Dev Biol 179:485-492, 1996.
204. Choi T, Fukasawa K, Zhou R, Tessarollo L, Borror K, Resau J, and Vande Woude GF: The Mos/MAP kinase pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. Proc Natl Acad Sci USA 93:7032-7035, 1996.
205. Taylor GA, Jeffers M, Largaespada DA, Jenkins NA, Copeland NG and Vande Woude GF: Identification of a novel GTPase, IGTP, that accumulates in response to interferon gamma. J Biol Chem 271:20399-20405, 1996.
206. Jeffers M, Rong S, Oskarsson M, Anver M, and Vande Woude GF: Autocrine hepatocyte growth factor/scatter factor signaling induces transformation and the invasive/metastatic phenotype in C127 cells. Oncogene 13:853-861,1996.
207. Webb CP, Lane K, Dawson AP, Vande Woude GF, and Warn RM: Characteristisation of a variant MDCK cell line that is non-responsive to HGF/SF but shows certain features consistent with constitutive met activation. J Cell Sci 109:2371-2381, 1996.
208. Jeffers M, Rao MS, Rulong S, Reddy JK, Subbarao V, Hudson E, Vande Woude GF and Resau JH: Hepatocyte growth factor/scatter factor-Met signaling induces proliferation, migration and morphogenesis if pancreatic oval cells. Cell Growth & Diff 7:1805-1813, 1996.
209. Fissore RA, He C-L and Vande Woude GF: Potential role of mitogen-activated protein (MAP) kinase during meiosis resumption in bovine oocytes. Biol of Reprod 55:1261-1270, 1996.
210. Jeffers M, Rong S and Vande Woude GF: Hepatocyte growth factor/scatter factor- Met signaling in tumorigenicity and invasion/metastasis. J Mol Med 74:505-513, 1996.
211. Koo H-M, Monks A, Mikheev A, Rubinstein LV, Gray-Goodrich M, McWilliams MJ, Alvord WG, Oie HK, Gazdar AF, Paull KD, Zarbl H, and Vande Woude GF. Enhanced sensitivity to cytosine arabinoside and topoisomerase II inhibitors in tumor cell lines harboring mutations in ras oncogenes. Cancer Res 56:5211-5216, 1996.
212. Jeffers M, Taylor GA, Weidner KM, Omura S and Vande Woude GF: Degradation of the met tyrosine kinase receptor by the ubiquitin-proteosome pathway. Mol Cell Biol 17:799-808, 1997.
213. Fukasawa K and Vande Woude GF: Synergy between Mos/MAPK oncogene activation and p53 inactivation in transformation and chromosome instability. Mol Cell Biol 17:506-518, 1997.
214. Murakami MS and Vande Woude GF: Mechanisms of Xenopus oocyte maturation. In: Methods in Enzymology 283:584-600, 1997.
215. Taylor GA, Stauber R, Rulong S, Hudson E, Pei V, Pavlakis GV, Resau JH and Vande Woude GF: The inducibly-expressed GTPase, IGTP, localizes to the endoplasmic reticulum, independently of GTP binding, J Biol Chem, 272:10639-10645, 1997.
216. Duesbery NS, Choi T, Brown KD, Wood K, Resau J, Fukasawa K, Cleveland DW and Vande Woude GF: CENP-E is masked in metaphase II-arrested mature oocytes. Proc Natl Acad Sci USA 94:9165-9170, 1997.
217. Murakami MS and Vande Woude GF: Analysis of the early embryonic cell cycles of Xenopus; regulation of cell cycle length by Xe-wee1 and Mos. Development 125:237-248, 1998.
218. Cortner J, Vande Woude S and Vande Woude GF: Mechanisms of Xenopus oocyte maturation. In: Advances in Veterinary Medicine, W.J. Dodds and J.E. Womack (eds). Vol. 40:51-102, 1997.
219. Fiscella M, Zhang H-L, Fan S, Sakaguchi K, Shen S, Mercer WE, Vande Woude GF, O'Connor PM and Appella E: Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. Proc Natl Acad Sci USA 94:6048-6053, 1997.
220. Fukasawa K, Wiener F, Vande Woude GF and Mai S: Genomic instability and apoptosis are frequent in p53 deficient young mice. Oncogene 15:1295-1302, 1997.
221. Jeffers M, Schmidt L, Nakaigawa N, Webb CP, Weirich G, Kishida T, Zbar B and Vande Woude GF: Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci USA

94:11445-11450, 1997.

222. Koochekpour S, Jeffers M, Rulong S, Klineberg E, Taylor G, Hudson EA, Resau JH and Vande Woude GF: Met and hepatocyte growth factor/scatter factor expression in human gliomas. Cancer Res 57:5391-5398, 1997.
223. Beilmann M, Odenthal M, Jung W, Vande Woude GF, Dienes H-P and Schirmacher P: Neoexpression of the c-met/hepatocyte growth factor-scatter factor receptor gene in activated monocytes. Blood 90:1-9, 1997.
224. Vande Woude GF, Jeffers M, Cortner J, Alvord G, Tsarfaty I, and Resau J: Met- HGF/SF: Tumorigenesis, Invasion and Metastasis. In: CIBA Foundation Symposium 212: Plasminogen-Related Growth Factors. GR Bock and JA Goode (Eds), John Wiley & Sons, Ltd., West Sussex, England, pp 119-132, 1997.
225. Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, Copeland TD, Ahn NG, Oskarsson, MK, Fukasawa K, Paull KD, and Vande Woude GF: Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280:734-737, 1998.
226. Webb CP, Van Aelst L, Wigler MH, and Vande Woude GF: Signaling pathways in ras-mediated tumorigenicity and metastasis. Proc Natl Acad Sci USA 95:8773-8778, 1998.
227. Taylor GA, Jeffers M, Webb CP, Koo H-M, Anver M, Sekiguchi K, Lechleider R, Roberts A, and Vande Woude GF: Decreased fibronectin expression in Met/HGF-mediated tumorigenesis. Oncogene 17:1179-1183,1998.
228. Webb CP, Taylor GA, Jeffers M, Fiscella M, Oskarsson M, Resau JH, and Vande Woude GF: Evidence for a role of Met-HGF/SF during ras-mediated tumorigenesis/metastasis. Oncogene 17:2019-2025, 1998.
229. Jeffers M, Koochekpour S, Fiscella M, Sathyanarayana BK, and Vande Woude GF: Signaling requirements for oncogenic forms of the Met tyrosine kinase receptor. Oncogene 17:2691-2700, 1998.
230. Jeffers M, Fiscella M, Webb CP, Anver M, Koochekpour S, and Vande Woude GF: The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci USA 95:14417-14422, 1998.
231. Duesbery N and Vande Woude GF: Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. In: Biology of the Cell, Y. Masui (ed), Vol. 90:461-466, 1998.
232. Schmidt L, Junker K, Weirich G, Glenn G, Choyke P, Lubensky I, Zhuang Z, Jeffers M, Vande Woude G, Neumann H, Walther M, Linehan WM, and Zbar B: Two North American families with hereditary papillary renal carcinoma and identical novel mutations in the MET proto-oncogene. Cancer Res 15:1719-22, 1998.
233. Koo, H-M, Gray-Goodrich M, Kohlhagen G, McWilliams MJ, Jeffers M, Vaigro-Wolff A, Alvord WG, Monks A, Paull K, Pommier Y, and Vande Woude GF: The ras oncogene sensitizes human cells to apoptosis induced by topoisomerase II inhibitors. J NCI 91:236-244, 1999.
234. Murakami M and Vande Woude GF: Mos positively regulates Xe-wee1 to lengthen the first mitotic cell cycle of Xenopus. Genes & Dev 13:620-631, 1999.
235. Ronen D, Alstock RT, Firon M, Mittelman L, Sobe T, Resau JH, Vande Woude GF and Tsarfaty I: Met-HGF/SF mediates growth arrest and differentiation in T47D breast cancer cells. Cell Growth & Diff 10:131-40, 1999.
236. Tsarfaty I, Resau J, Alstock RT, Lidereau R, Bieche I, Bertrand F, Horev J, Alvord WG, Keydar I and Vande Woude GF: Alteration of met expression and prognosis in breast cancer. Analyt and Quant Cyt and Hist 21:397-408, 1999.
237. Jeffers M and Vande Woude GF: Activating mutations in the met receptor overcome the requirement for autophosphorylation of tyrosines crucial for wild type signaling. Oncogene 18:5120-5125, 1999.
238. Duesbery N and Vande Woude GF: Anthrax lethal factor. In Cell Mol Life Sci 55:1599-1609, 1999.
239. Ried S, Jager C, Jeffers M, Vande Woude GF, Graeff H, Schmitt M and Lengyel E: Activation mechanisms of the urokinase-type plasminogen activator promoter by hepatocyte growth factor/scatter factor (HGF/SF). J Biol Chem 274:16377-16386, 1999.
240. Duesbery NS, Webb CP and Vande Woude GF: MEK wars, a new front in the battle against cancer. Nature Med News & Views 5:736-737, 1999.
241. Koochekpour S, Jeffers M, Wang PH, Gong C, Taylor GA, Roessler LM, Stearman R, Edwards DR, Stetler-Stevenson WG, Kaelin Jr. WG, Linehan WM, Gnarra J, Klausnser RD and Vande Woude GF: Hepatocyte growth factor/scatter factor (HGF/SF) induces von Hippel-Lindau (VHL) dependent invasion

and branching morphogenesis in renal carcinoma cells. Mol Cell Biol, 19:5902-5912, 1999.

242. Duesbery N and Vande Woude GF: Anthrax lethal factor causes proteolytic inactivation of MAP-Kinase-Kinase. Lett in Appl Micro, 87:289-293, 1999.
243. Beilmann M , Vande Woude GF, Dienes H-P, Schirmacher P: Hepatocyte growth factor-stimulated invasiveness of monocytes. Blood 95:2964-3969, 2000.
244. Webb CP, Hose CD, Koochekpour S, Jeffers M, Oskarsson M, Sausville E, Monks A, and Vande Woude GF: The geldanamycins are potent inhibitors of the HGF/SF-Met-uPA-plasmin proteolytic network. Cancer Res 60:342-349, 2000.
245. Koo HM, McWilliams MJ, Alvord WG, and Vande Woude GF: Ras oncogene-induced sensitization to 1-beta-D-arabinofuranosylcytosine. Cancer Res 59:6057-6062, 1999.
246. Taylor GA, Collazo CM, Yap GS, Nguyen K, Gregorio TA, Taylor LS, Eagleson B, Secrest L, Southen EA, Reid SW, Tessarollo L, Bray M, McVicor DW, Komsclies KL, Young HA, Biron CA, Sher A, and Vande Woude GF: Pathogen specific loss of host resistance in mice lacking the interferon-gamma-inducible gene IGTP. Proc Natl Acad Sci USA 97:751-755, 2000.
247. Taylor GA and Vande Woude GF: Regulation of P311 expression by Met-HGF/SF and the ubiquitin/proteasome system. J Biol Chem 275:4215-4219, 2000.
248. Lee JH, Han S, Cho H, Jennings B, Gerrard B, Dean M, Schmidt L, Zbar B, and Vande Woude GF: A novel germ line juxtamembrane Met mutation in human primary gastric cancer. Oncogene 19:4947-4953, 2000.
249. Vousden K and Vande Woude GF: The ins and outs of p53, Nature Cell Biology News & Views, Volume 2, E178-E180, 2000. Furge K, Zhang Y-W, and Vande Woude GF: Met receptor tyrosine kinase: Enhanced signaling through adapter proteins. Oncogene Review 19:5582-5589, 2000.
250. Webb CP and Vande Woude GF: Genes that regulate metastasis and angiogenesis. J Neuro-Oncology 50:71-87, 2000.
251. Smith JS, Evans EK, Murakami M, Moyer MB, Mosely MA, Vande Woude G and Kornbluth S: Wee1-regulated apoptosis mediated by the Crk adaptor protein in Xenopus egg extracts. J Cell Biol 151:1391-1400, 2000.
252. Xiao G-H, Jeffers M, Bellacosa A, Mitsuuchi Y, Vande Woude GF, and Testa JR: Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and MAP kinase pathways. Proc Natl Acad Sci USA 98:247-252, 2001. Webb CP and Vande Woude GF: Animal model for Ras-induced metastasis. In Methods in Enzymology. WE Balch, CJ Der and A Hall (eds). Academic Press, San Diego, 318-329, 2001.
253. Hammond DE, Urbe' S, Vande Woude GF, and Clague MJ: Down-regulation of MET, the receptor for hepatocyte growth factor. Oncogene 20:2761-2770, 2001. Duesbery NS, Resau J, Webb CP, Koochekpour S, Koo H-M, Leppla SH, and Vande Woude GF: Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. Proc Natl Acad Sci USA 98:4089-4094, 2001.
254. Cao B, Su Y, Oskarsson M, Zhao P, Kort, EJ, Fisher RJ, Wang L-M, and Vande Woude GF: Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models. Proc Natl Acad Sci USA 98:7443-7448, 2001. Collazo CM, Yap GS, Sempowski GD, Lusby KC, Tessarollo L, Vande Woude GF,
255. Sher A and Taylor GA: Inactivation of LRG-47 and IRG-47 reveals a family of interferon γ-inducible genes with essential, pathogen-specific roles in resistance to infection. J Exp Med 194:181-187, 2001.
256. Gmyrek GA, Walburg MN, Webb CP, Yu H-M, You X, Vaughan ED, Vande Woude GF and Knudsen BS: Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. Am J Pathol 159:579-590, 2001.
257. Furge KA, Kiewlich D, Le P, Vo MN, Lipson KE, Faure M, Howlett AR, Vande Woude GF and Webb CP: Suppression of Ras-mediated tumorigenicity and metastasis through inhibition of the Met receptor tyrosine kinase. Proc Natl Acad Sci 98:10722-10727, 2001.
258. Furge K and Vande Woude GF: Met. In Encyclopedic Reference of Cancer. M Schwab (Ed.). Springer-Verlag, Heidelberg, pp 550-553, 2001.

259. Zhang Y-W, Wang L-M, Jove R and Vande Woude GF: Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. Oncogene 21:217-226, 2002.

260. Hay RV, Cao B, Skinner RS, Wang L-M, Su Y, Resau JH, Vande Woude GF and Gross MD: Radioimmunoscintigraphy of tumors autocrine for human Met and hepatocyte growth factor/scatter factor. Mol Imaging 1:56-62, 2002.

261. Koo H-M, McWilliams MJ, VanBrocklin M, Azucena EF, Leppla SH, Duesbery NS, and Vande Woude GF: Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. PNAS 99:3052-3057, 2002.

262. Qian C-N, Guo X, Cao B, Kort EJ, Lee C-C, Chen J, Wang L-M, Mai W-Y, Min H-Q, Hong M-H, Vande Woude GF, Resau JH, and Teh BT: Met protein expression level correlates with survival in patients with late-stage nasopharyngeal carcinoma. Cancer Res 62:589-596, 2002.

263. Webb CP and Vande Woude GF: Met gene. In Wiley Encyclopedia of Molecular Medicine, Vol. 3. H H Kazazian, Jr. (ed). Wiley, New York, 2049-2051 (2002). Duesbery NS and Vande Woude GF: An arresting activity. Nature News & Views 416:1-2, 2002.

264. Frankel AE, Powell BL, Duesbery NS, Vande Woude GF, Leppla SH: Anthrax fusion protein therapy of cancer. Curr Protein Pept Sci 3:399-407, 2002.

265. Koo H-M, Duesbery NS, and Vande Woude GF: Anthrax toxins, mitogen-activated protein kinase pathway, and melanoma treatment. Directions in Science 1:123-126, 2002.

266. Knudsen BS, Gmyrek GA, Inra J, Scherr DS, Darracott VE, Nanus DM, Kattan MW, Gerald WL, and Vande Woude GF: High expression of the Met receptor in prostate cancer metastasis to bone. Urology 60:1113-7, 2002.

267. Hay R, Cao B, Tsarfaty I, Tsarfaty G, Resau J, Vande Woude, GF: Grappling with metastatic risk: Bringing molecular imaging of MET expression toward clinical use. J Cell Biochem 39:184-193, 2002

268. Zhang Y-W and Vande Woude GF: HGF/SF Met signaling in the control of branching morphogenesis and invasion. J Cell Biochem 88:408-17, 2003. Hay R, Cao B, Skinner RS, Wang L, Su Y, Resau J, Knudsen B, Gustafson M,

269. Koo H-M, Vande Woude GF, and Gross M: Radioimmunoscintigraphy of human Met-expressing tumor xenografts using Met3, a new monoclonal antibody Clin Can Res 9:3839-3844, 2003.

270. Hammond DE, Carter S, McCullough J, Urbe' S, Vande Woude G, and Clague MJ: Endosomal dynamics of Met determine signaling output, Mol Biol Cell 14:1346-1354, 2003.

271. You X, Yu H-M, Cohen-Gould L, Cao, B, Symons M, Vande Woude GF, and Knudsen BS: Regulation of migration of primary prostate epithelial cells by secreted factors from prostate stromal cells. Exp Cell Res 288:246-256, 2003.

272. Frankel AE, Koo H-M, Leppla SH, Duesbery NS and Vande Woude GF: Novel protein targeted therapy of metastatic melanoma. Curr Pharm Design 9:2060-2066, 2003.

273. Shinomiya N and Vande Woude GF: Suppression of Met expression: A possible Cancer treatment. Commentary on "Reduced c-Met expression by an adenovirus expressing a c-Met ribozyme inhibits tumorigenic growth and lymph node metastases of PC3-LN4 prostate tumor cells in an orthotopic nude mouse model" (Kim et al.). Clin Can Res 14:5161-5170, 2003.

274. Zhang Y-W, Su Y, Volpert OV, and Vande Woude GF: HGF/SF mediates angiogenesis through positive VEGF and negative thrombospondin-1 regulation. Proc Natl Acad Sci USA 100:12718-12723, 2003.

275. Hay RV, Cao B, Skinner RS, Gustafson M, Su Y, Qian CN, The BT, Knudsen BS, Resau J, Vande Woude G, and Gross MD: Met5, a new monoclonal antibody for radioimmunoscintigraphy of Met-expressing tumors. J Nuclear Med 44:178, 2003.

276. Birchmeier C, Birchmeier W, Gherardi E, and Vande Woude GF: Met, metastasis, motility and more. Nature Rev Mol Cell Biol 4:915-925, 2003.

277. Fan J, Tam P, Vande Woude G and Ren Y: Normalization and significant analysis of cDNA micro-arrays using within-array replications applied to neuroblastoma cell response to MIF. Proc Natl Acad Sci USA 101:1135-40, 2004.

278. Vande Woude GF, Kelloff GJ, Ruddon RW, Koo H-M, Sigman CC, Barrett JC, Day RW, Dicker AP, Kerbel RS, Parkinson DR, and Slichenmyer WJ: Reanalysis of cancer drugs: Old drugs, new tricks. Clin Cancer

Res 10:3897-3907, 2004. Kelloff GJ, Bast RC Jr, Coffey DS, D'Amico AV, Kerbel RS, Park JW, Ruddon RW, Rustin GJS, Schilsky RL, Sigman CC, and Vande Woude GW: Biomarkers, surrogate end points, and the acceleration of drug development for cancer prevention and treatment: An update. Clin Cancer Res 10:3881-3884, 2004

279. Lee CC, Putnam AJ, Miranti CK, Gustafson M, Wang LM, Vande Woude GF, and Gao CF: Overexpression of sprouty 2 inhibits HGF/SF-Mediated cell growth, invasion, migration and cytokinesis. Oncogene 23:5193-5202 2004.
280. Zhang Y-W, Graveel C, Shinomiya N, and Vande Woude, GF: Met decoys: Will cancer take the bait? Cancer Cell Previews 5-6, July 2004.
281. Shinomiya N, Gao CF, Xie Q, Gustafson M, Zhang, Y-W, Waters DJ, and Vande Woude GF: RNA interference reveals that ligand-independent Met activity is required for tumor cell signaling and survival. Cancer Res 64:7962-7970, 2004.
282. Lengyel E, Prechtel D, Resau JH, Gauger K, Welk A, Lindemann K, Salanti G, Richter T, Knudsen B, Vande Woude GF, and Harbeck N. c-Met overexpression in node- positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. Int J Cancer 113:678-82, 2004.
283. Graveel C, Su Y, Koeman J, Wang L-M, Tessarollo L, Fiscella M, Birchmeier C, Swiatek P, Bronson R, Vande Woude G: Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci USA 101:17198-17203, 2004.
284. Islam A, Sakamoto Y, Kosaka K, Yoshitome S, Sugimoto I, Yamada K, Shibuya E, Vande Woude GF and Hashimoto E: The distinct stage-specific effects of 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid on the activation of MAP kinase and Cdc2 kinase in Xenopus oocyte maturation". Cellular Signaling 17:507-523, 2005.
285. Zhang Y-W, Su Y, Lanning N, Gustafson M, Shinomiya N, Zhao P, Cao B, Tsarfaty G, Wang L-M, Hay R, and Vande Woude GF: Enhanced growth of human Met-expressing xenografts in a new strain of immunocompromised mice transgenic for human HGF/SF. Oncogene 6:101-106, 2005.
286. Gao CF and Vande Woude GF: HGF/SF-Met signaling in tumor progression. Cell Res. 15:49-51, 2005.
287. Xie Q, Gao CF, Shinomiya N, Sausville E, Hay R, Gustafson M, Shen Y, Wenkert D, and Vande Woude GF. Geldanamycins exquisitely inhibit HGF/SF-mediated tumor cell invasion. Oncogene, in press, 2005.
288. Hay RV, Cao B, Skinner RS, Su Y, Zhao P, Gustafson MF, Qian C-N, Teh BT, Knudsen BS, Resau JH, Shen S, Waters DJ, Gross MD, Vande Woude GF: Nuclear imaging of Met-expressing human and canine xenografts with radiolabeled monoclonal antibodies (MetSeekTM). Clin Can Res, in press, 2005.
289. Graveel CR, London CA, and Vande Woude GF: Extra views: A mouse model of activating met mutations. Cell Cycle, in press, 2005. Jiao Y, Zhao P, Zhu J, Grabinski T, Feng Z, Guan X, Skinner RS, Gross MD, Su Y, Vande Woude GF, Hay RV, and Cao B: Construction of human naïve Fab library and characterization of anti-Met Fab fragment generated from the library. Molec Biotech, in press, 2005.

Atty Dkt: 29025.0001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Arlene RAMSINGH *et al.*

Serial No.: 09/879,572

Filing Date: June 12, 2001

For: COXSACKIEVIRUS B4 EXPRESSION VECTORS AND USES THEREOF

Examiner: Stacy Chen

Group Art Unit: 1648

Atty Dkt: 29025.0001

Customer No. 26694

PATENT TRADEMARK OFFICE

3RD DECLARATION OF ARLENE I. RAMSINGH PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, the undersigned, declare as follows:

1. I am a co-inventor of the present application. My *Curriculum Vitae* and background have already been submitted as part of an earlier Declaration. It is my intention here to help clarify the record in this case by recounting some relevant history of Coxsackievirus B4 (CB4) viruses and the variants that are described in this application, well-known in the art, and that are intended to be used in the claimed invention.

2. The prototypic strain of CB4, JVB, was isolated in the 1950's and deposited in the ATCC by Sickles and Daldorf from the institute which was the forerunner of my place of employment, the Wadsworth Center of the New York State Dept. of Public Health. This deposited virus was given ATCC # VR-184 and remains publicly available. It can be found by entering this number on the ATCC virus search page

http://www.atcc.org/common/catalog/animalVirology/animalVirologyIndex.cfm .

3. Virus derived from the deposited JVB strain has been sequenced at least twice, first by Jenkins and colleagues (Jenkins *et al.* 1987[1]) and then by my lab - half the genome - from the 5' end (Ramsingh *et al.* 1992[2]). The latter sequence is also in GenBank, Accession #S39291 (see: http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=250908). The sequence differences between CB4 viruses are discussed below.

4. Origin of the CB4-P designation: I was given the JVB strain of CB4 by a colleague, R. Diebel, at the Wadsworth Center in 1989 and "renamed it CB4-P" to stand for the prototype CB4 virus which was intended to distinguish it from its virulent variant, CB4-V. Thus CB4-P is JVB, despite the fact that the sequences are not identical, as explained below. A paper by my group published in 1989[3] was the first to describe what we now call "CB4-P." This paper states at p. 349 that Coxsackie B4 (JVB) was kindly provided by R. Diebel (Wadsworth Center) On p. 350, it states that the prototypical CB4 (JVB) is designated CB4-P. The (-P) designation distinguishes this virus from a virulent one designated CB4-V. CB4-P was not to be distinguished from JVB - rather I considered it the same and merely renamed it.

5. Random Variation/Spontaneous Mutation in Viruses: As virus populations are propagated, they inexorably accumulate random mutations and begin to diverge somewhat from the "parental" sequence. RNA virus populations are constantly changing due to the lack of editing function in the enzyme that replicates their genes, RNA-dependent RNA polymerase. Thus, RNA virus populations are "plastic" and this plasticity leads to the ongoing generation of genetic variants within a strain. See Declaration of Steven Tracy (submitted concurrently). Many of these mutations are ***neutral*** either because they do not result in amino acid changes, or, if they do, the change does not affect function so there is no selection for or against them. The net result is that if the original deposited JVB virus from the 1950's had been sequenced and compared to the JVB virus that the ATCC would have sent out in 1987 or 1992 or 2004 under the same ATCC #, some changes in nucleotide, as well as in amino acid, sequence would have been inevitable as a result of

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[1] Jenkins *et al.* 1987. "The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the picornaviridae." *J. Gen. Virol. 68*:1835-1848)

[2] Ramsingh *et al.* 1992. "Identification of candidate sequences that determine virulence in Coxsackievirus B4." *Virus Res 23*:281-292

[3] Ramsingh *et al.* 1989. "Severity of disease induced by a pancreatropic Coxsackie B4 virus correlates with the H-2Kq locus of the major histocompatibility complex. *Virus Res 14*:347-358

growing the virus (in the ATCC lab or the recipient's lab). Such virus, if stored and then retested (sequenced) years later (even under the same strain designation) would have those few mutations generated during the previous rounds of cultivation. This fully explains the nucleotide sequence differences between JVB in the virus population of Jenkins et al. (*supra)* and CB4-P (Ramsingh et al., 1992, *supra*). Unless the environment in which the virus was passaged had been radically changed (different cell types, temperatures, multiplicities-of-infection, etc.), one would expect to find a virus (in 2004, for example, but derived from the much earlier ATCC deposit) to be very closely related, with only a small number of changes in the sequence. The virus population at these two points in time would be considered the same virus (=the same viral strain).

6. Sequence Differences: The two sequences for the JVB/CB4-P virus were found to be ever so slightly different. Again, this variability arose from random mutation during passage.

(A) A comparison of the amino acid sequences of several picornaviruses (the class to which coxsackieviruses belong) performed by Jenkins *et al. (supra)* showed that the P1 region encoding the viral polyprotein (and, through it, the four viral capsid proteins) was the most divergent. Comparison of the P1 regions of two distinct **serotypes** of the Coxsackie group B viruses, CB3 and CB4, showed 77.9% nucleotide sequence identity. CB4 and CB3 are *different* virus populations (despite the high degree of sequence identity).

(B) A comparison of the sequences of the P1 regions of the JVB virus reported by Jenkins *et al. (supra)* and the CB4-P virus (also JVB), reported by Ramsingh et al., 1992, *supra*, reveals 99.8% at the nucleotide level and 99.6% at the amino acid level. Therefore, CB4-P and the JVB are essentially the same virus population. In a stretch of almost 3300 nt's, that includes the P1 region - the most divergent/variable part of the viral genome - there were only 9 total nucleotide substitutions (4 non-coding and 5 coding regions). Two of the coding region mutations were silent whereas the other 3 resulted in amino acid substitutions which did not affect viral function (and bear **no relationship** to the site of, or nature of, the genetic manipulations of the present invention). This variation is exceedingly small so that two such sequences would never be considered "heterologous".

If I were to fully sequence nucleic acid of the CB4-P virus stock I have stored in my lab now (with or without further propagation), there would likely be some random variation in sequence from the same virus that we sequenced in 1992. It would still be the same virus (or a genetic equivalent). The reasons for this were explained above. It is my practice to always verify the sequence of the relevant region of the virus (where heterologous DNA is to be inserted) when preparing a new virus batch. Those skilled in the art would do the same, thereby confirming that they have the same virus.

7. The current Office Action states that CB4-P is "required to practice the claimed invention" which I understand to mean that the invention cannot be practiced by another person unless that person is handed the CB4-P virus that I used. This leads the Patent Office to conclude that the application does not adequately support such use without a Patent Deposit of this virus. This statement and conclusion is not accurate for several reasons.

(A) Between the application and the publicly available sequence information, including that in Jenkins *et al., supra*; Ramsingh et al., 1992, *supra* and Caggana *et al.*, 1993, (which is part of record of this case and cited in the present Office Action), a person of ordinary skill in the field can routinely make, test (verify) and use the genetically engineered virions (or nucleic acid molecules) of the claims - that comprise an exogenous, foreign, heterologous nucleotide sequence inserted in the indicated sites of the P1 region of the viral genome. The person can start with any CB4 virus that is described in the application or known in the field, and *if desired*, can modify the viral nucleic acid sequence to make it match up perfectly with the CB4-P virus that I described in the application and whose sequence I reported years earlier. However, importantly, as would be appreciated by a person of skill in this field, there is no reason the starting virus would have to match up perfectly over its entire genome. It just needs to have the correct sequences at the sites used to introduce the heterologous nucleic acid, and that is described in detail in the specification.

(B) Technologically speaking, any virus that is a genetic equivalent of CB4-P could readily be used to practice the claimed invention. The point of genetic equivalence is important because we are dealing here with RNA viruses. As noted in Section 5, above, and in the accompanying Tracy Declaration, RNA virus populations are constantly changing. CB4-P is one genetic variant of the JVB strain of CB4. Genetic variants of a strain are genetically

equivalent viruses because they share a high degree of sequence identity. As an example, the nucleotide sequence of the most divergent region of the viral genome, P1, of CB4-P (as reported in Ramsingh *et al.* 1992, *supra*) and that of JVB (as reported in Jenkins *et al.* *supra*) shows 99.8% sequence identity while the amino acid sequences are 99.6% identical.

8. The specification teaches insertion of heterologous nucleic acids into the genome of CB4 using CB4-P as but a convenient example. The salient point for appreciating the scope of the invention is the genetic equivalence and not the phenotypic equivalence (*i.e.*, virulent vs. less virulent or avirulent) because genetically equivalent viruses can be easily manipulated by those skilled in this field, as described in the specification, to allow insertions of heterologous nucleic acids. The deposited JVB strain of CB4 (ATCC # VR-184) is genetically equivalent to CB4-P. Any CB4 virus, including the deposited JVB strain, can be used to practice the claimed invention. The specification goes into the functional similarity between CB4-P and JVB on page 6, lines 13-15; and the equivalency of CB4-P and JVB on page 14, lines 8-15

> One example of such an attenuated B4 coxsackievirus is J.V.B. (Benschoten), ATCC reference number 184 (referred to herein as JVB). The prototype virus CB4-P is originally derived from JVB and is highly similar to JVB in nucleotide and amino acid sequence. Because of this strong conservation, the JVB virus is expected to perform as an equivalent to CB4-P in the generation and use of the viral vector described herein.

9. To conclude, the CB4 virus - - the starting material for the claimed recombinant coxsackievirus B4 virions[4] which are engineered to contain an inserted heterologous nucleic acid that encodes a heterologous polypeptide fused to a capsid protein of the virion - - is publicly available. Also available, in the form of published papers, GenBank sequences and the present application, is all the information needed to make and use the claimed virions and nucleic acid composition. On that basis, the Patent Office's position that people in the field would not be enabled to practice the invention fully is in error and the requirement that a patent deposit of "CB4-P" virus be made is unnecessary.

[4] and for the claimed nucleic acid molecules

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

May 26, 2005 Arlene I. Ramsingh

Date Arlene I. Ramsingh

J. gen. Virol. (1987), **68**, 1835–1848. *Printed in Great Britain*

Key words: *coxsackievirus B4/nucleotide sequence/picornavirus*

The Complete Nucleotide Sequence of Coxsackievirus B4 and Its Comparison to Other Members of the Picornaviridae

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SUMMARY

The genome of the prototype strain of coxsackievirus B4 (J.V.B. Benschoten) has been cloned in *Escherichia coli* and its complete nucleotide sequence determined. Excluding the poly(A) tract, the RNA genome is 7395 nucleotides in length and appears to encode a single polyprotein of 2183 amino acids. The predicted amino acid sequence of the polyprotein shows close homology (88%) to that of the previously sequenced coxsackievirus B3 and to certain regions of the polyproteins of the polioviruses and human rhinovirus 14. This allows identification of putative polyprotein cleavage signals, antigenic domains and other structural features likely to be important to the biological integrity of the virus.

INTRODUCTION

Coxsackieviruses are members of the enterovirus genus of the family Picornaviridae. They are divided into 23 group A serotypes (CA1 to CA22, CA24) and six group B serotypes (CB1 to CB6). This antigenically diverse group of viruses has a limited host range, but exhibits a broad spectrum of tissue tropism within the natural host and is associated with a correspondingly large variety of clinical illnesses, ranging from mild respiratory infections to severe myocarditis and neurological disorders (Grist *et al.*, 1978; Melnick, 1985). The group B coxsackieviruses and especially CB4 are of particular interest in that they have been associated with type 1 juvenile onset or insulin-dependent diabetes mellitus (Yoon *et al.*, 1979; King *et al.*, 1983; Barret-Connor, 1985; Frisk *et al.*, 1985). The molecular basis of the tissue tropism of these viruses is not well understood, although there is some evidence to suggest that for the picornaviruses generally, a major determinant is the affinity of the viruses for a specific cellular receptor on the surface of the target cell (see Crowell *et al.*, 1981).

Knowledge of the primary structure and genetic organization of picornaviruses has increased dramatically in recent years, and this has provided insights into the mechanisms of replication (Kitamura *et al.*, 1981), genetic relationships (Stanway *et al.*, 1984*a*), evolution (Toyoda *et al.*, 1984), antigenicity (Minor *et al.*, 1983, 1986) and pathogenicity (Stanway *et al.*, 1984*b*; Cann *et al.*, 1984; La Monica *et al.*, 1986; Evans *et al.*, 1985). All picornaviruses share common structural features, namely an approximately 30 nm capsid of icosahedral symmetry, made up of 60 copies each of four virus-coded proteins (VP1 to VP4) enclosing a single-stranded, positive-sense RNA genome of approximately 7500 nucleotides. The RNA is polyadenylated at its 3′ terminus and has a small protein, VPg, covalently attached to the 5′ terminus. The primary translation product of picornavirus RNA is a single large polyprotein which is processed by virus-encoded proteases to yield the mature viral proteins (for a review, see Rueckert, 1985).

Recently the complete nucleotide sequence of CB3 (strain Nancy) has been determined (Lindberg *et al.*, 1987), providing detailed information on coxsackievirus genome organization and allowing a comparison at the nucleotide level with other members of the Picornaviridae.

As part of a study into the molecular basis of serotype diversity, pathogenicity and tissue tropisms of these viruses, we have determined the complete nucleotide sequence of the genome

of the prototype strain of CB4 (J.V.B. Benschoten). We provide a detailed comparison of this sequence with that of the closely related CB3 and with those of other members of the Picornaviridae. The high degree of similarity to poliovirus (PV) type 1 and rhinovirus 14 (HRV14) also allows interpretation of the predicted amino acid sequence data in relation to the three-dimensional structures recently determined for these two viruses (Hogle *et al.*, 1985; Rossmann *et al.*, 1985).

METHODS

Virus and cells. Coxsackievirus B4 (strain J.V.B. Benschoten) (Dalldorf, 1950) was obtained from the American Type Culture Collection. The virus was propagated in HEp-2c cells and purified on sucrose gradients as previously described (Minor, 1980).

Molecular cloning and nucleotide sequencing. Purified viral RNA (approximately 2 μg) was reverse-transcribed and cloned into *Escherichia coli* JA221 by the cDNA:RNA hybrid method (Cann *et al.*, 1983; Stanway *et al.*, 1984*c*) using vector pBR322. Of the recombinants obtained, approximately 1000 were screened by hybridization using 3′-enriched and randomly primed CB4 cDNA probes (Cann *et al.*, 1983). Plasmid DNA isolated from strongly hybridizing colonies was further characterized by restriction enzyme mapping and by cross-hybridization. A set of five overlapping clones were selected which together spanned the genome (data not shown). The sequences of cDNA inserts were determined by the dideoxynucleotide method after generation of random fragments and cloning into M13mp8 as previously described (Stanway *et al.*, 1984*b*). This method was used to obtain the nucleotide sequence of the majority of the genome. The sequence of the remainder was obtained after cloning specific restriction fragments into M13mp18 or mp19. The whole of the sequence was determined at least twice and approximately 75% of it was obtained in both orientations. Throughout its assembly the sequence was compared to those of other enteroviruses and this provided a useful check on possible frameshift sequencing errors. Where significant differences were observed these were checked in the opposite orientation. The sequence data were assembled and analysed using published computer programs (Staden, 1980).

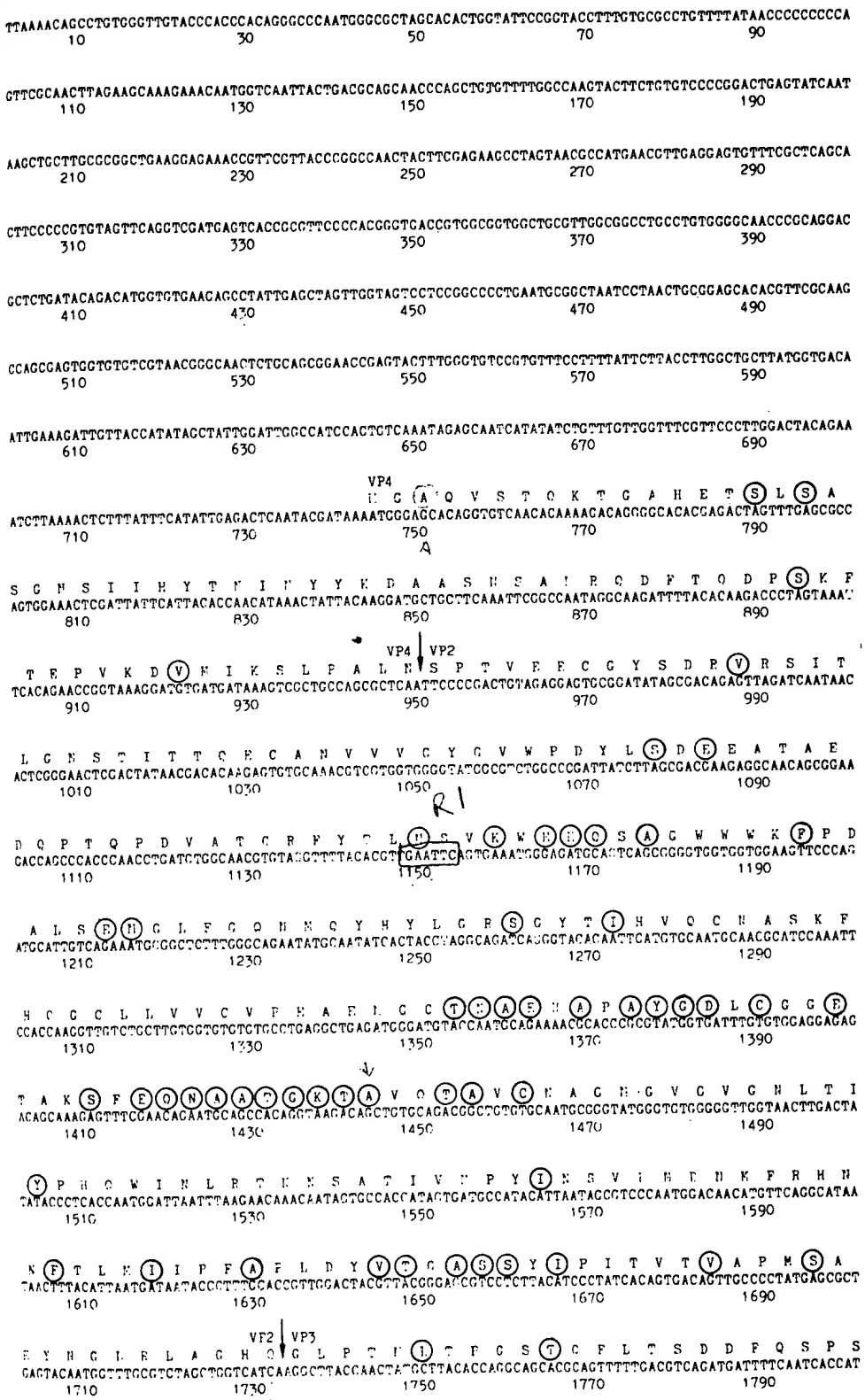
RESULTS AND DISCUSSION

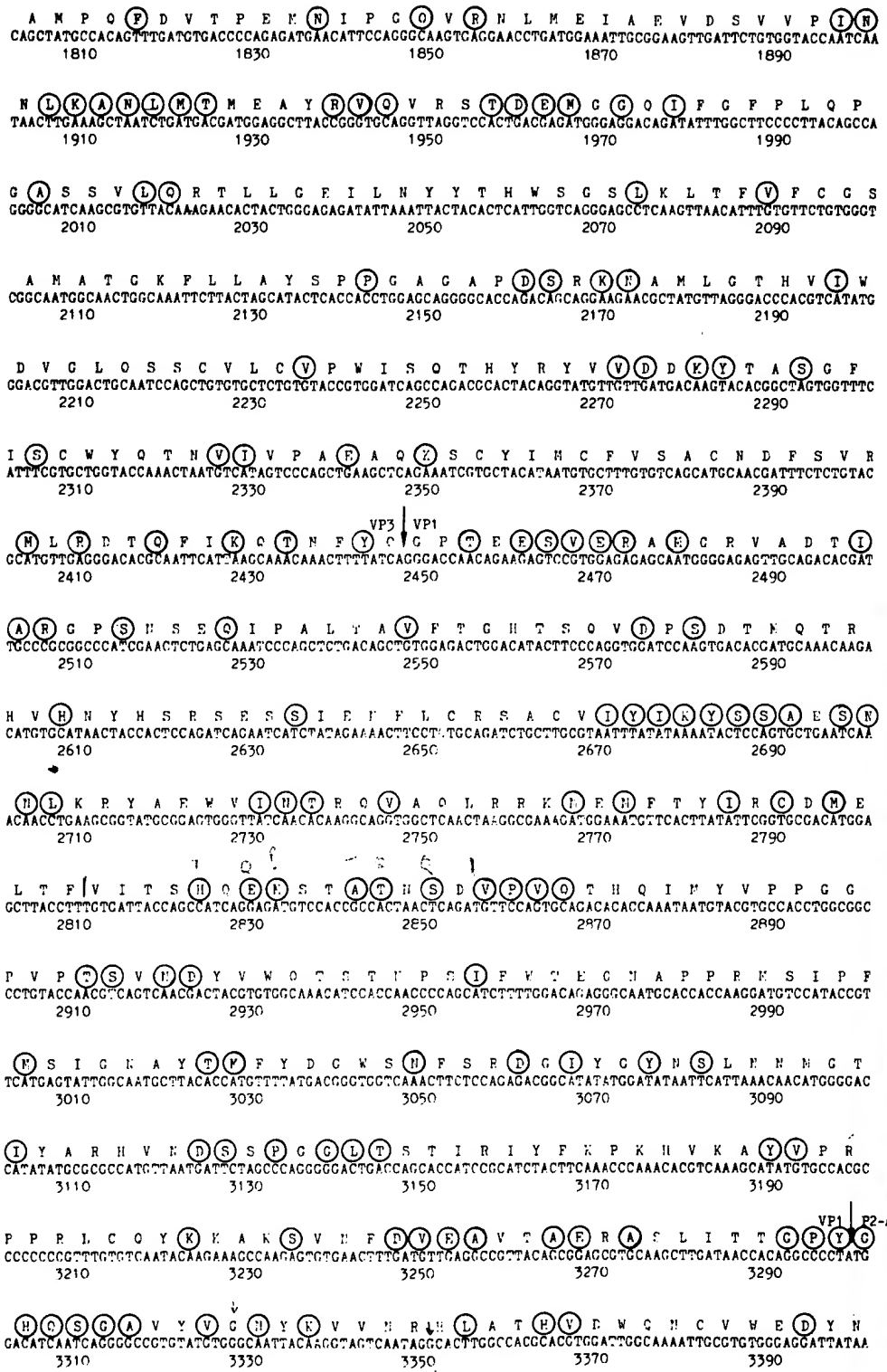
The complete nucleotide sequence and predicted amino acid sequence of CB4 (strain J.V.B. Benschoten) are shown in Fig. 1. The genome is highly homologous to those of other enteroviruses, suggesting that it has a similar genetic organization. Thus, the genome comprises a 5′ non-coding region of 743 nucleotides, a single open reading frame of 6552 nucleotides (2184 codons) and a 3′ non-coding region of 100 nucleotides prior to a poly(A) tract. Hence the total size of the CB4 genome excluding the poly(A) tract is 7395 nucleotides (compared with CB3, 7396; PV1, 7433; PV3, 7432; HRV14, 7208 nucleotides) and it has the % base composition A, 28·30; G, 24·84; C, 22·91; T, 23·95.

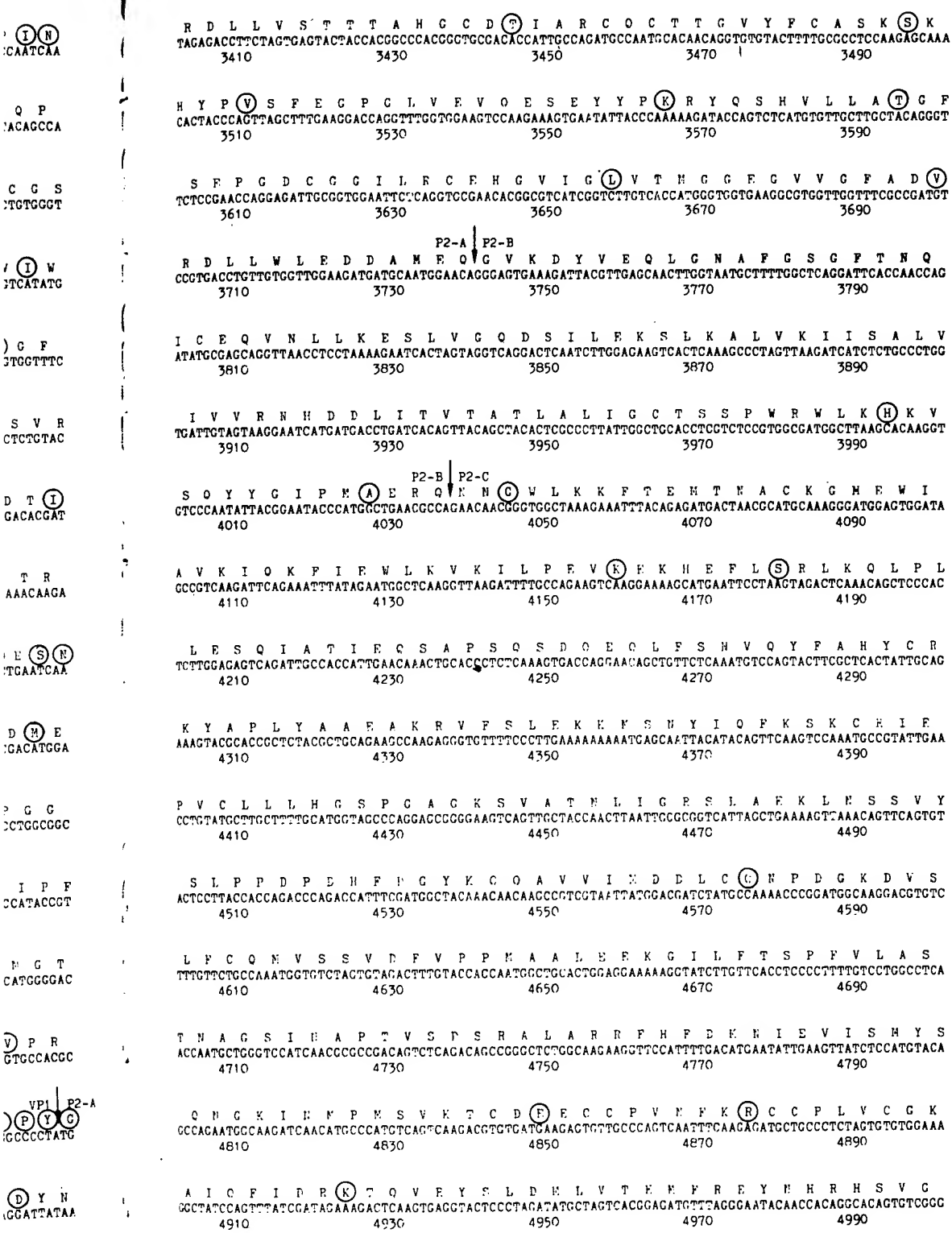
5′ non-coding region

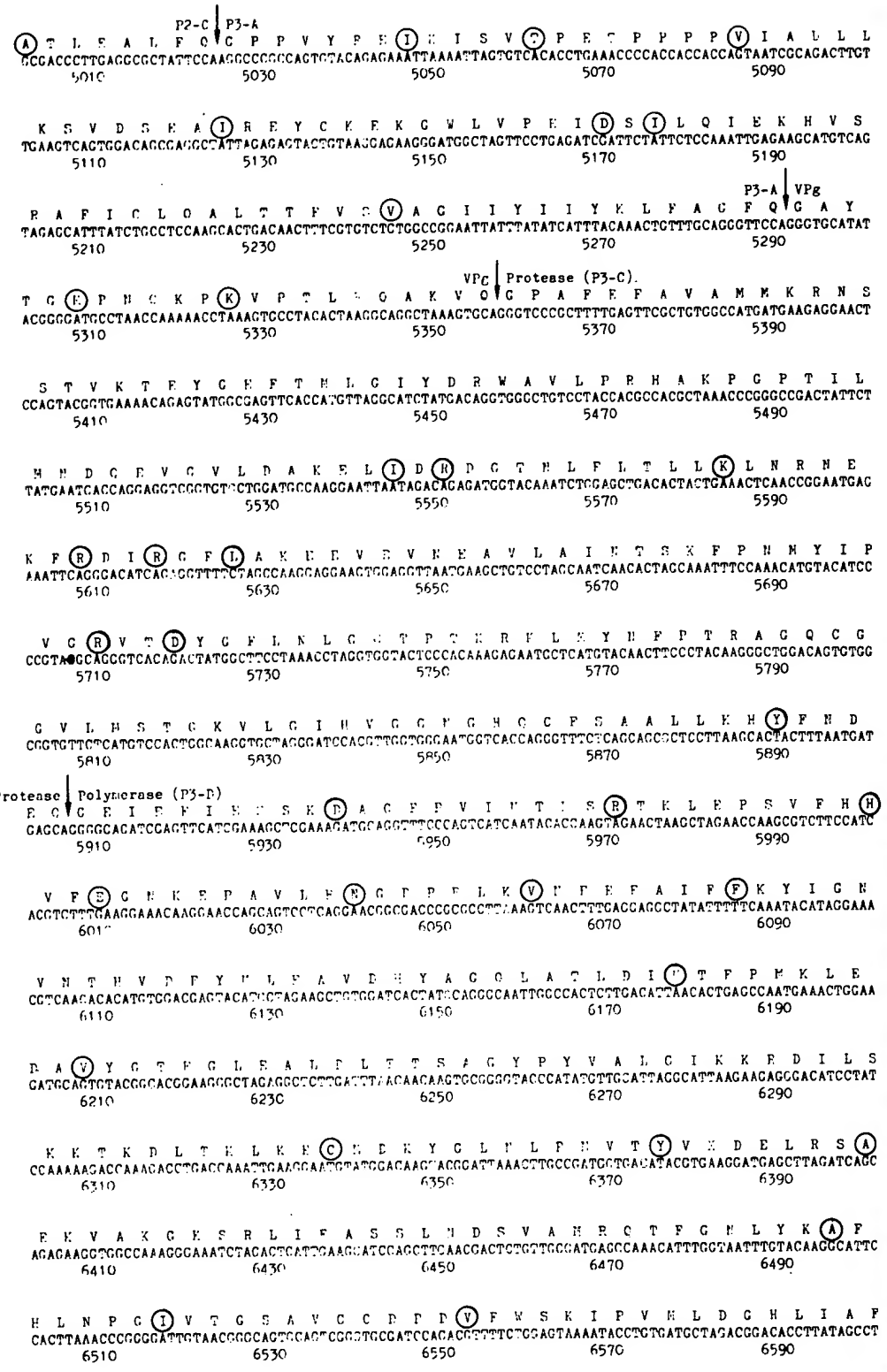
By analogy with the polioviruses, the 5′ terminal 743 nucleotides of the CB4 genome are assumed to be non-coding (Kitamura *et al.*, 1981). There are, however, seven potential translation start codons prior to that which initiates the large open reading frame at position 744. Four of these are quickly followed by stop codons. Those at positions 272 and 463 are followed by open reading frames of 66 codons and 110 codons respectively. The latter AUG is also present in CB3 and PV3 but the size of the reading frame is not conserved, being terminated after 76 amino acids at position 686 in CB3 and after 44 amino acids at position 593 in PV3. These similarities may be fortuitous though it is likely that they relate to sequence-dependent functions other than translation. It is considered unlikely that any of these short open reading frames are translated since the corresponding peptides have not been found in PV-infected cells. Furthermore, none of the AUGs have flanking nucleotide sequences favoured by eukaryotic ribosomes for the initiation of protein synthesis (Kozak, 1986).

The 5′ non-coding regions of CB4, CB3, PV1, PV3 and HRV14 can be aligned by taking into account several small deletions or insertions to give remarkable sequence homology (Table 1, Fig. 2). The first 10 nucleotides are identical in all these viruses. This conservation of sequence may be important for interactions with proteins involved in the replication of the viral RNA (Lindberg *et al.*, 1987; Hewlett & Florkiewicz, 1980; Toyoda *et al.*, 1984). Other regions of pronounced conservation include nucleotide positions 66 to 85, 446 to 472 and 547 to 567 and


VP4
VP4 VP2
R1
VP2 VP3


VP3
VP1
VP1
P2-A


R D L L V S T T T A H G C D T I A R C Q C T T G V Y F C A S K S K
TAGAGACCTTCTAGTGAGTACTACCACGGCCCACGGGTGCCACACCATTGCCAGATGCCAATGCACAACAGGTGTGTACTTTTGCGCCTCCAAGAGCAAA
3410 3430 3450 3470 3490
H Y P V S F E C P C L V E V Q E S E Y Y P K R Y Q S H V L L A T G F
CACTACCCAGTTAGCTTTGAAGGACCAGGTTTGGTGGAAGTCCAAGAAAGTGAATATTACCCAAAAAGATACCAGTCTCATGTGTTGCTTGCTACAGGGT
3510 3530 3550 3570 3590
S F P G D C G G I L R C E H G V I G L V T M G G E G V V G F A D V
TCTCCGAACCAGGAGATTGCGGTGGAATTCTCAGGTCCGAACACGGCGTCATCGGTCTTGTCACCATGGGTGGTGAAGGCGTGGTTGGTTTCGCCGATGT
3610 3630 3650 3670 3690
P2-A | P2-B
R D L L W L E D D A M E Q ↓ G V K D Y V E Q L G N A F G S G F T N Q
CCGTGACCTGTTGTGGTTGGAAGATGATGCAATGGAACAGGGAGTGAAAGATTACGTTGAGCAACTTGGTAATGCTTTTGGCTCAGGATTCACCAACCAG
3710 3730 3750 3770 3790
I C E Q V N L L K E S L V C Q D S I L E K S L K A L V K I I S A L V
ATATGCGAGCAGGTTAACCTCCTAAAAGAATCACTAGTAGGTCAGGACTCAATCTTGGAGAAGTCACTCAAAGCCCTAGTTAAGATCATCTCTGCCCTGG
3810 3830 3850 3870 3890
I V V R N H D D L I T V T A T L A L I G C T S S P W R W L K H K V
TGATTGTAGTAAGGAATCATGATGACCTGATCACAGTTACAGCTACACTCGCCCTTATTGGCTGCACCTCGTCTCCGTGGCGATGGCTTAAGCACAAGGT
3910 3930 3950 3970 3990
P2-B | P2-C
S Q Y Y G I P M A E R Q ↓ K N C W L K K F T E M T N A C K G M E W I
GTCCCAATATTACGGAATACCCATGGCTGAAGGCCAGAACAACGGGTGGCTAAAGAAATTTACAGAGATGACTAACGCATGCAAAGGGATGGAGTGGATA
4010 4030 4050 4070 4090
A V K I Q K F I E W L K V K I L P E V K E K H E F L S R L K Q L P L
GCCGTCAAGATTCAGAAATTTATAGAATGGCTCAAGGTTAAGATTTTGCCAGAAGTCAAGGAAAAGCATGAATTCCTAAGTAGACTCAAACAGCTCCCAC
4110 4130 4150 4170 4190
L E S Q I A T I E Q S A P S Q S D Q E Q L F S H V Q Y F A H Y C R
TCTTGGAGAGTCAGATTGCCACCATTGAACAAACTGCACCTCTCAAAGTGACCAGGAAGAGCTGTTCTCAAATGTCCAGTACTTCGCTCACTATTGCAG
4210 4230 4250 4270 4290
K Y A P L Y A A E A K R V F S L E K K F S N Y I Q F K S K C E I E
AAAGTACCCACCGCTCTACGCTGCAGAAGCCAAGAGGGTGTTTTCCCTTGAAAAAAAAATGAGCAATTACATACAGTTCAAGTCCAAATGCCGTATTGAA
4310 4330 4350 4370 4390
P V C L L L H G S P G A G K S V A T N L I G R S L A E K L N S S V Y
CCTGTATGCTTGCTTTTGCATGGTAGCCCAGGAGCCGGGAAGTCAGTTGCTACCAACTTAATTGCGCGGTCATTAGCTGAAAAGTCAAACAGTTCAGTGT
4410 4430 4450 4470 4490
S L P P D P D H F D G Y K C Q A V V I N D D L C G N P D G K D V S
ACTCCTTACCACCAGACCCAGACCATTTCGATGGCTACAAACAACAAGCCGTCGTAATTATGGACGATCTATGCCAAAACCCGGATGGCAAGGACGTGTC
4510 4530 4550 4570 4590
L F C Q N V S S V D F V P P M A A L E E K G I L F T S P F V L A S
TTTGTTCTGCCAAATGGTGTCTAGTGTAGACTTTGTACCACCAATGGCTGCACTGGAGGAAAAAGGTATCTTGTTCACCTCCCCTTTTGTCCTGGCCTCA
4610 4630 4650 4670 4690
T N A G S I N A P T V S D S R A L A R R F H F D K N I E V I S H Y S
ACCAATGCTGGGTCCATCAACCGCCCACAGTCTCAGACAGCCGGGCTCTGGCAAGAAGGTTCCATTTTGACATGAATATTGAAGTTATCTCCATGTACA
4710 4730 4750 4770 4790
VP1 | P2-A
Q N G K I N N P M S V K T C D F E C C P V N F K R C C P L V C G K
GCCAGAATGGCAAGATCAACATGCCCATGTCAGTCAAGACGTGTGATGAAGAGTGTTGCCCAGTCAATTTCAAGAGATGCTGCCCTCTAGTGTGTGGAAA
4810 4830 4850 4870 4890
A I C F I D R K T Q V E Y S L D E L V T E E F R E Y N H R H S V C
GCCTATCCAGTCTATCGATAGAAAGACTCAAGTGAGGTACTCCCTAGATATGCTAGTCACGGAGATGTTTAGGGAATACAACCACAGGCACAGTGTCGGG
4910 4930 4950 4970 4990


P2-C | P3-A
P3-A | VPg
VPg | Protease (P3-C).
Protease | Polymerase (P3-D)

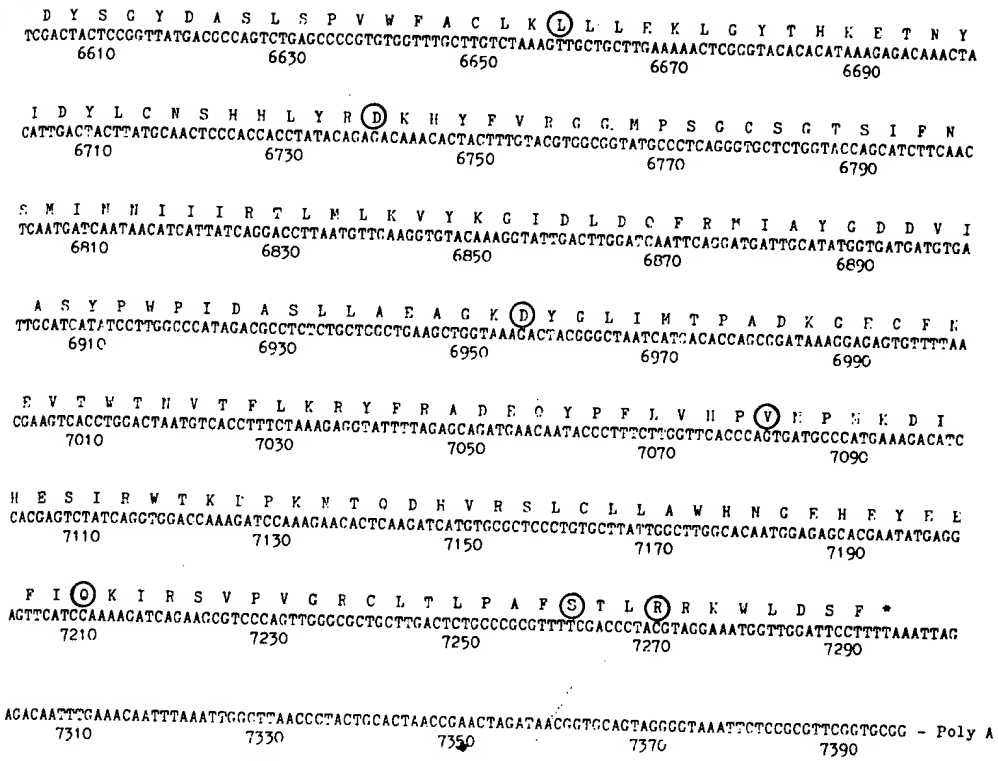


Fig. 1. The complete nucleotide sequence and predicted amino acid sequence of the polyprotein of CB4 (J.V.B. Benschoten). Amino acid differences from the previously sequenced CB3 (Nancy), are circled. Predicted polyprotein cleavage sites are arrowed.

Table 1. *Nucleotide sequence homologies between the non-coding regions of CB4 and four other picornaviruses**

	CB4 : CB3	CB4 : PV1	CB4 : PV3	CB4 : HRV14
5′ non-coding	84·4	71·4	71·1	62·8
3′ non-coding	93·8	59·1	59·1	45·2

* Sequence homologies are expressed as percentages. Details of the other virus sequences taken from Lindberg *et al.* (1987) for CB3, Kitamura *et al.* (1981) for PV1, Stanway *et al.* (1984*b*) for PV3 and Stanway *et al.* (1984*a*) for HRV14.

these correspond to those previously described as highly conserved between HRV14 and PV3 (Stanway *et al.*, 1984*a*). It has been suggested that these conserved sequences may be important for RNA secondary structure which is necessary for an, as yet, unspecified function in replication (Tracy *et al.*, 1985; Toyoda *et al.*, 1984; Newton *et al.*, 1985). Of interest in this respect is a region from nucleotides 10 to 34 which in the polioviruses has the potential to form a stable stem–loop secondary structure (Larsen *et al.*, 1981; Stanway *et al.*, 1983). In the corresponding region, CB4 is not well conserved in primary sequence but can form a similar stem–loop structure composed of exactly the same number of GC and AT base pairs. This stem–loop is likely to have functional importance in replication since it has been shown for PV2 that deletion of base 10 (which destabilizes the structure) leads to a mutant virus with a temperature-sensitive phenotype. This mutation appears to affect the rate of protein synthesis at both permissive and non-permissive temperatures (Racaniello & Meriam, 1987). Interestingly the corresponding regions in HRV14 and HRV2 do not appear to form such structures readily,

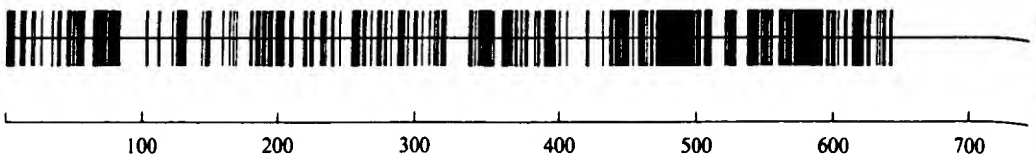


Fig. 2. Comparison between the 5′ non-coding regions of CB4 (J.V.B. Benschoten), CB3 (Nancy), PV1 (Mahoney), PV3 (P3/Leon/37) and HRV14. Vertical bars represent conserved nucleotides. Nucleotide numbers (Fig. 1) are indicated.

Table 2. *Proposed proteolytic cleavage sites in the polyproteins of five picornaviruses**

Boundary	CB4	CB3	PV1†	PV3	HRV14
VP4/VP2	N/S	N/S	N/S	N/S	N/S
VP2/VP3	Q/G	Q/G	Q/G	Q/G	Q/G
VP3/VP1	Q/G	Q/G	Q/G	Q/G	Q/T? Q/T? E/G?
VP1/P2-A	Y/G	Q/S? I/R? Y/R? Q/N?	Y/G	Y/G	Y/G? Y/G?
P2-A/P2-B	Q/G	Q/G	Q/G	Q/G	Q/G
P2-B/P2-C	Q/N	Y/G? Q/N?	Q/G	Q/G	Q/A
P2-C/P3-A	Q/G	Q/G	Q/G	Q/G	Q/G
P3-A/VPg	Q/G	Q/G	Q/G	Q/G	Q/G
VPg/protease (P3-C)	Q/G	Q/G	Q/G	Q/G	Q/G
Protease/polymerase (P3-D)	Q/G	Q/G	Q/G	Q/G	Q/G

* Details taken from Tracy *et al.* (1985) and Lindberg *et al.* (1987) for CB3, Stanway *et al.* (1984*b*) for PV3 and Stanway *et al.* (1984*a*) for HRV14.

† Cleavage sites in PV1 were determined by amino acid sequencing (Larsen *et al.*, 1982; Pallansch *et al.*, 1984).

suggesting that the function is not indispensable. The hundred or so nucleotides prior to the initiation of translation of the polyprotein, as in the polioviruses, are poorly conserved and it is unlikely that this region has any functional significance, although it has been suggested that it may play a role in the conservation of the length of the 5′ non-coding region (Toyoda *et al.*, 1984). This idea does not hold for the rhinoviruses, however, where the region appears to have been completely deleted.

Translated region

Translation of the CB4 RNA probably initiates at nucleotide 744. The initiation codon at this position forms part of the sequence AAAAUGG, which is an almost optimal translation initiation sequence for eukaryotic ribosomes (Kozak, 1986). In this frame, there are no termination codons until nucleotide 7293. The region therefore can encode a polyprotein of 2183 amino acids, consistent with the known replication strategy of the picornaviruses (see Rueckert, 1985). The amino acid homology between the predicted proteins of CB4 and those of CB3, PV1, PV3 and HRV14 are shown in Table 3 and represented diagrammatically in Fig. 3. The homology with PV1, for which a detailed genetic map has been determined biochemically (Pallansch *et al.*, 1984; Kuhn & Wimmer, 1987) facilitates the identification of the sites in the CB4 polyprotein at which the virus-encoded proteases are likely to act. These are presented in Table 2 together with the assumed cleavage sites of CB3, PV3, HRV14, and the determined cleavage sites of PV1. With the exception of that at the P2-B/P2-C junction all of the cleavage sites in PV1 are conserved in CB4 and are therefore likely to be utilized. To identify the likely cleavage site between P2-B and P2-C, a comparison with CB3 is helpful. Lindberg *et al.* (1987)

Table 3. *Amino acid sequence homology between the proteins of CB4 and four other picornaviruses**

Protein	CB4:CB3	CB4:PV1	CB4:PV3	CB4:HRV14
VP4	94·2	69·6	71·0	60·8
VP2	80·5	56·0	56·3	54·6
VP3	78·2	55·9	55·9	46·8
VP1	71·2	47·3	44·6	35·7
Total P1	77·9	54·4	54·1	47·9
P2-A	91·2	57·8	59·1	44·4
P2-B	97·9	50·5	51·5	55·7
P2-C	97·6	62·6	62·9	58·7
P3-A	92·1	47·1	50·6	48·2
VPg	90·9	77·3	72·7	40·9
Protease (P3-C)	95·0	60·7	60·6	52·7
Polymerase (P3-D)	95·2	74·0	73·9	65·9

* Sequence homologies are expressed as percentages. Details taken from Lindberg *et al.* (1987) for CB3, Kitamura *et al.* (1981) for PV1, Stanway *et al.* (1984*b*) for PV3 and Stanway *et al.* (1984*a*) for HRV14.

have proposed that in CB3 this cleavage occurs either at a tyrosine–glycine pair or a glutamine–asparagine pair which by alignment correspond to those in CB4 at nucleotide positions 4013 and 4037 respectively (Fig. 1, Table 2). In PV1 this cleavage occurs at a glutamine–glycine pair which by alignment corresponds exactly to the glutamine–asparagine pair in CB4. The amino acid sequence following this site is highly conserved between CB4, CB3 and PV1, whereas the amino acid sequence around the tyrosine–glycine pair (although conserved between CB4 and CB3) shows no homology to the corresponding region in the polioviruses. Moreover glutamine–asparagine has been shown by amino acid sequencing to be a cleavage site in HRV2 (Skern *et al.*, 1985). We conclude therefore that the glutamine–asparagine pair probably functions as the cleavage site for P2-B/P2-C in both CB3 and CB4.

Of interest also is the P1/P2 cleavage site occurring between a tyrosine and glycine residue in HRV14, and all serotypes of poliovirus (Table 2). The sequence comparisons indicate that this site is conserved in CB4 and is therefore likely to be used, but that it is not present in CB3 (Fig. 1, Table 2). Four alternative sites have been proposed for the corresponding cleavage in CB3 (Tracy *et al.*, 1985; Lindberg *et al.*, 1987) (Table 2). Unfortunately the two coxsackieviruses are very different in this region and the CB4 sequence does not therefore help to distinguish between the four possibilities for cleavage in CB3.

Cleavage of the poliovirus precursor polypeptide VP0 to give VP4 and VP2 occurs at an asparagine–serine amino acid pair and is thought to be an autocatalytic event (Hogle *et al.*, 1985). This site is also conserved at an equivalent position in the CB4 polyprotein (nucleotide position 950) and is likely therefore to function in a similar manner (Fig. 1).

Verification of these proposed polyprotein processing sites in CB4 would, of course, require N- and C-terminal protein sequence analysis of infected cell and virus structural polypeptides. However, the high degree of homology to the other enteroviruses in these regions of the polyprotein provides persuasive evidence that the cleavage sites identified in Table 2 for CB4 are correct. The sizes of the predicted CB4 proteins are summarized in Table 4.

Structural proteins

The P1 region of the polyprotein forms the precursor to the four structural proteins VP1 to VP4. As expected, and in common with the rhinoviruses and most other enteroviruses, VP4 is the most conserved of the capsid proteins. In PV1 and HRV14, VP4 is not exposed on the outer surface of the virion (Hogle *et al.*, 1985; Rossmann *et al.*, 1985) and does not contribute to antigenic domains important in virus neutralization (Minor *et al.*, 1986). This protein is therefore unlikely to be subjected to immune selection pressure. In the three other structural proteins, VP1, VP2 and VP3, there are regions that are highly conserved, interspersed with regions of substantial divergence (Fig. 3). Regions of highest homology are those likely to be of importance in maintaining the stable secondary structure of the capsid proteins and these

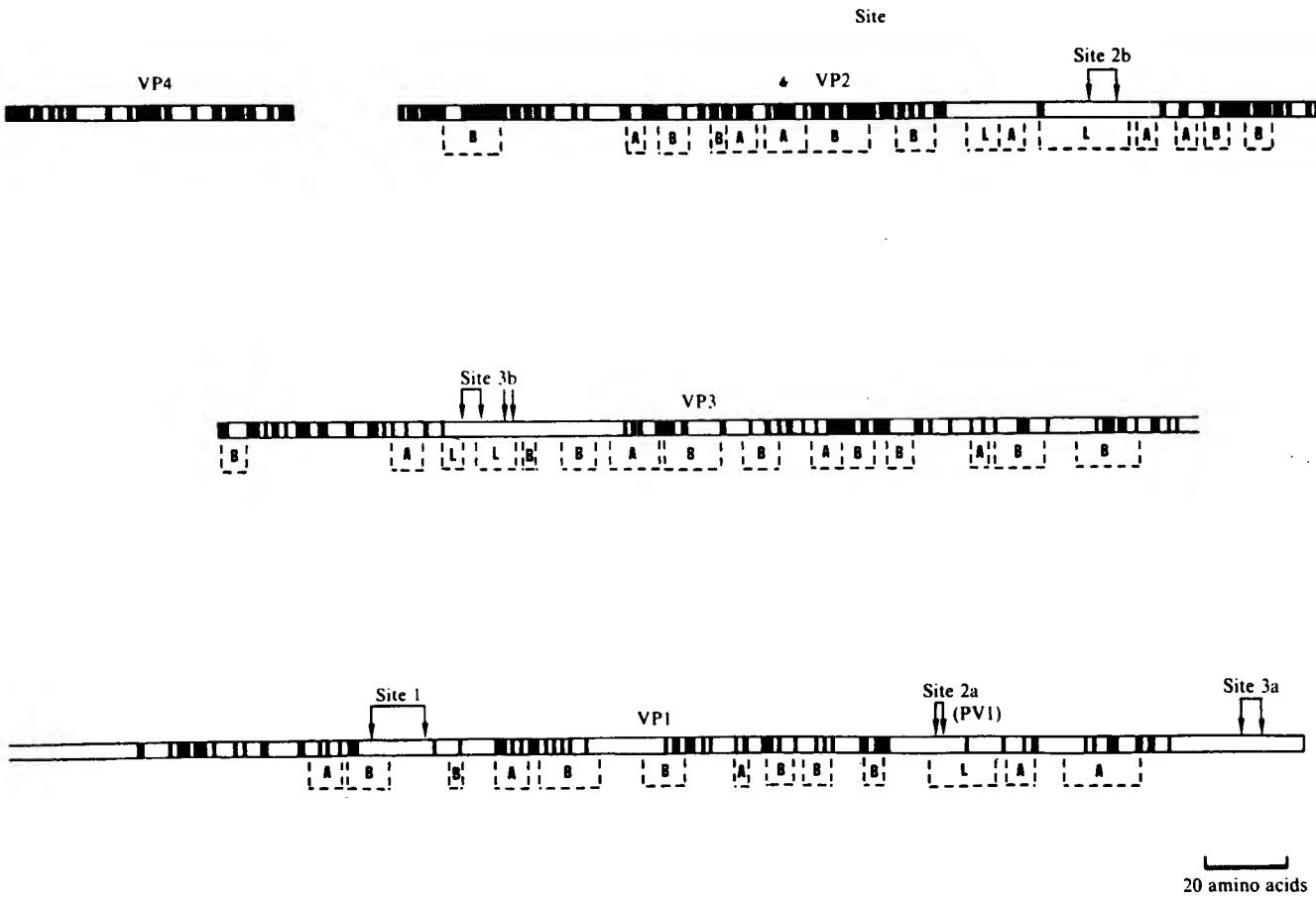


Fig. 3. Comparison between the capsid proteins of CB4 (J.V.B. Benschoten), CB3 (Nancy), PV1 (Mahoney), PV3 (P3/Leon/37) and HRV14. Conserved amino acids are represented by black boxes. Antigenic sites determined for PV3 are shown. Regions identified in the 3-D structure of PV1 as α-helices (A), β-sheets (B) and random coil or loop-out regions (L) are indicated.

Table 4. *CB4 proteins and their sizes**

Protein	Number of amino acids	Mol. wt. ($\times 10^{-3}$)
VP4	69	7·49
VP2	261	28·56
VP3	238	26·43
VP1	284	31·98
P2-A	147	16·22
P2-B	99	10·96
P2-C	329	37·27
P3-A	89	9·96
VPg	22	2·41
Protease (P3-C)	183	20·33
Polymerase (P3-D)	462	52·53
Total polyprotein	2183	243·96

* Based on the cleavage sites proposed in Table 2.

correspond, by alignment with PV1, to α-helices and β-sheets in the three-dimensional structure (Hogle *et al.*, 1985). By contrast, the regions which show least homology correspond to the more flexible loop-out or random coil secondary structures, some of which are known to be antigenic determinants in polioviruses and rhinoviruses (Minor *et al.*, 1986; Rossmann *et al.*, 1985; Skern *et al.*, 1987; Sherry & Rueckert, 1985; Fig. 3). Thus it is likely that in CB4 the amino acids 76 to 87 in VP1, 158 to 164 in VP2 and 58 to 60 and 70 to 71 in VP3 are antigenically important. Although other amino acids are also likely to contribute to antigenicity, the imprecision of the alignment in these regions of random coil makes their identification less reliable.

Non-structural proteins

The P2 region is the part of the polyprotein most highly conserved between the two coxsackieviruses B3 and B4. There are only two amino acid differences beween them in the P2-B protein and only eight differences in the P2-C protein (Fig. 1, Table 3). The functions of both of these proteins are unknown, but there is evidence that P2-C forms part of the membrane-bound replication complex (Takegami *et al.*, 1983). Mutations conferring guanidine resistance in PV1 have also been mapped to this protein (Pincus *et al.*, 1986). P2-A has been shown to function as a protease responsible for cleavage of the polyprotein at tyrosine–glycine cleavage sites (Toyoda *et al.*, 1986). As discussed above, the amino acid pair tyrosine–glycine is present at the P1/P2 junction in CB4, PV1, PV3 and HRV14 (Table 2) but is not present in CB3. It is therefore interesting to note that P2-A of CB4 is substantially more homologous to the P2-A of CB3 (91%) even though their substrates are different, than to those of the other viruses (less than 60%) in which the substrate cleavage site is the same.

The P3 region is processed to give P3-AB (which is subsequently processed to provide VPg), a protease (P3-C) and an RNA-dependent RNA polymerase (P3-D). The VPgs of coxsackieviruses B1, B3 and B5 have been compared at the amino acid level (Lindberg *et al.*, 1987). The VPg sequence of CB4 differs from those of CB1 and CB5 by only one amino acid, and differs from the CB3 sequence by two. These changes are conservative. As is the case in all enteroviruses both the protease (P3-C) and polymerase (P3-D) of coxsackieviruses are highly conserved (Fig. 1, Table 3), the polymerase being the most highly conserved protein of those specified by picornavirus genomes (Table 3; Argos *et al.*, 1984).

3′ non-coding region

The translation of the polyprotein is terminated at position 7293 by the sequence UAA which is followed by a non-coding region of 100 nucleotides prior to the poly(A) tract (Fig. 1). This region is very highly conserved between CB4 and CB3 (94%, Table 1) and also between the three serotypes of polioviruses (98%, Toyoda *et al.*, 1984) but among the enteroviruses and rhinoviruses as a whole it is far less well conserved than the 5′ non-coding region. The function of this 3′ non-coding region in picornaviruses has not yet been determined, although it is likely to

(*a*)

```
                1                                                    50
CB4     aattagagac aatttgaaac aatttaaatt ggcttAACCC TACtgca..c
CB3     gattagagac aa.ttgaaac aatttagatt ggcacAACCC TACtg..tgC
PV3     .......... .......... .......... ...gtAACCC TACctcagtC
Consens ---------- ---------- ---------- -----AACCC TAC------C

               51                                                   100
CB4     tAAccGaAct aGaTaAcggT gcaGTAGGGG TAAATTcTcC gcgttcggtG
CB3     tAAccGaAcc aGaTaAcggT acaGTAGGGG TAAATTcTcC gcattcggtG
PV3     gAAttGgAtt gGgTcAtacT gttGTAGGGG TAAATTtTtC tttaattcgG
Consens -A--G-A-- -G-T-A---T ---GTAGGGG TAAATT-T-C ---------G

               101
CB4     cGg
CB3     cG.
PV3     aG.
Consens -G-
```

(*b*)

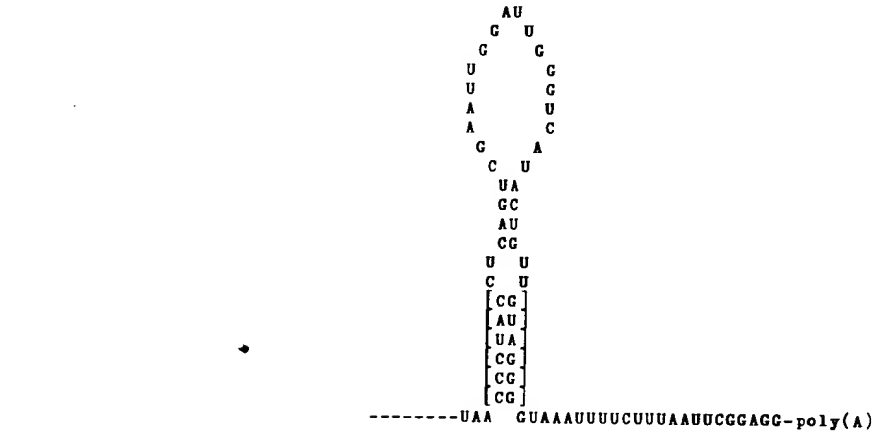


Fig. 4. (*a*) Alignment of the 3′ non-coding regions of CB4 (J.V.B. Benschoten), CB3 (Nancy) and PV3 (P3/Leon/37). Conserved nucleotides are shown in upper case. (*b*) Predicted RNA secondary stem–loop structure of this region. Conserved nucleotides underlined in (*a*) form part of the stem.

be involved in the control of genome replication (Fellner, 1979). The conserved blocks of nucleotides reported previously following comparisons of CB3, PV1 and swine vesicular disease virus (Stalhandske *et al.*, 1984) are also present in CB4 (Fig. 4). A number of different stem–loop secondary structures can be compiled for this region (Ryan, 1985) in which the conserved blocks of nucleotides form part of the stem. Occasionally sequence divergence within the stem is observed (e.g. in enterovirus 70; M. Ryan, unpublished), but base pairing is always maintained by compensatory mutations across the stem. It has been shown that the insertion of an eight nucleotide linker at position 7387 in the 3′ non-coding region of PV1 gives rise to a virus with a temperature-sensitive phenotype suggesting that the structure of the region has some essential function. The temperature-sensitive phenotype may result from destabilization of the secondary structure at the restrictive temperature (Sarnow *et al.*, 1986). It is interesting that HRV14 and HRV2 have much smaller 3′ non-coding regions than the enteroviruses. The conserved block of nucleotides closest to the poly(A) tract (Fig. 4) is deleted and it is therefore unlikely that the rhinoviruses are able to form similar secondary RNA structures. The significance of this observation is unclear, but it is interesting to note the parallel with the 5′ non-coding region as discussed above where the loop formed by nucleotides 10 to 34 is also missing in the rhinoviruses. These features may be consistent differences between the rhinoviruses and enteroviruses (Stanway *et al.*, 1984*a*).

It is clear that CB4 is very closely related to CB3 and to other members of the enterovirus genus. This high level of homology, particularly with the better studied members of the Picornaviridae such as PV1 and HRV14, allows a comprehensive interpretation of the sequence to be made. Thus, polyprotein cleavage signals can be confidently located and likely antigenic domains tentatively identified. These comparisons are useful in that they also indicate regions of sequence difference which must ultimately determine the characteristic biological properties which differ between picornaviruses. The possibility to manipulate these regions via site-directed mutagenesis of cloned cDNA provides an experimental approach to understanding the molecular basis of picornavirus diversity. Such experiments are in progress.

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REFERENCES

ARGOS, P., KAMER, G., NICKLEN, M. J. H. & WIMMER, E. (1984). Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest a common ancestry of these virus families. *Nucleic Acids Research* **12**, 7251–7267.

BARRET-CONNOR, E. (1985). Is insulin-dependent diabetes mellitus caused by coxsackievirus B infection? A review of the epidemiologic evidence. *Review of Infectious Diseases* **7**, 207–215.

CANN, A. J., STANWAY, G., HAUPTMANN, R., MINOR, P. D., SCHILD, G. C., CLARKE, L. D., MOUNTFORD, R. C. & ALMOND, J. W. (1983). Poliovirus type 3: molecular cloning of the genome and nucleotide sequence of the region encoding the protease and polymerase proteins. *Nucleic Acids Research* **11**, 1267–1281.

CANN, A. J., STANWAY, G., HUGHES, P. J., MINOR, P. D., EVANS, D. M. A., SCHILD, G. C. & ALMOND, J. W. (1984). Reversion to neurovirulence of the live-attenuated Sabin type 3 oral poliovirus vaccine. *Nucleic Acids Research* **12**, 7787–7792.

CROWELL, R. L., LANDAU, B. J. & SIAK, J. S. (1981). Picornavirus receptors in pathogenesis. In *Virus Receptors*, part II, pp. 169–184. Edited by K. Lonberg-Holm & L. Philipson. London: Chapman & Hall.

DALLDORF, G. (1950). The coxsackie viruses. *Bulletin of the New York Academy of Medicine* **26**, 329–335.

EVANS, D. M. A., DUNN, G., MINOR, P. D., SCHILD, G. C., CANN, A. J., STANWAY, G., ALMOND, J. W., CURREY, K. & MAIZEL, J. V. (1985). Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. *Nature, London* **314**, 548–550.

FELLNER, P. (1979). General organisation and structure of the picornavirus genome. In *The Molecular Biology of Picornaviruses*, pp. 25–48. Edited by R. Perez-Bercoff. New York: Plenum Press.

FRISK, G., FOHLMAN, J., KOBBAH, M., EWALD, U., TUVEMO, T., DIDERHOLM, H. & FRIMAN, G. (1985). High frequency of coxsackie-B-virus specific IgM in children developing type 1 diabetes during a period of high diabetes morbidity. *Journal of Medical Virology* **17**, 219–227.

GRIST, N. R., BELL, E. J. & ASSAAD, F. (1978). Enteroviruses in human disease. *Progress in Medical Virology* **24**, 114–157.

HEWLETT, M. J. & FLORKIEWICZ, R. Z. (1980). Sequence of picornavirus RNAs containing a radioiodinated 5′ linked peptide reveals a conserved 5′ sequence. *Proceedings of the National Academy of Sciences, U.S.A.* **77**, 303–307.

HOGLE, J. M., CHOW, M. & FILMAN, D. J. (1985). The three-dimensional structure of poliovirus at 2·9 Å resolution. *Science* **229**, 1358–1365.

KING, M. L., SHAIKH, A., BIDWELL, D., VOLLER, A. & BANATVALA, J. E. (1983). Coxsackie B virus specific IgM responses in children with insulin dependent (juvenile-onset; type 1) diabetes mellitus. *Lancet* i, 1397–1399.

KITAMURA, N., SEMLER, B. L., ROTHBERG, P. G., LARSEN, G. R., ADLER, C. J., DORNER, A. J., EMINI, E. A., HANECAK, R., LEE, J. J., VAN DER WERF, S., ANDERSON, C. W. & WIMMER, E. (1981). Primary structure, gene organization and polypeptide expression of poliovirus RNA. *Nature, London* **291**, 547–553.

KOZAK, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283–292.

KUHN, R. J. & WIMMER, E. (1987). The replication of picornaviruses. In *The Molecular Biology of Positive Stranded RNA Viruses*. Edited by D. J. Rowlands, B. W. J. Mahy & M. A. Mayo. London & Orlando: Academic Press (in press).

LA MONICA, N., MERIAM, C. & RACANIELLO, V. R. (1986). Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. *Journal of Virology* **57**, 515–525.

LARSEN, G. R., SEMLER, B. L. & WIMMER, E. (1981). Stable hairpin structure within the 5′-terminal 85 nucleotides of poliovirus RNA. *Journal of Virology* **37**, 328–335.

LARSEN, G. R., ANDERSON, C. W., DORNER, A. J., SEMLER, B. L. & WIMMER, E. (1982). Cleavage sites within the poliovirus capsid protein precursors. *Journal of Virology* **41**, 340–344.

LINDBERG, A. M., STALHANDSKE, P. O. K. & PETTERSSON, U. (1987). Genome of coxsackievirus B_3. *Virology* **156**, 50–63.

MELNICK, J. L. (1985). Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In *Virology*, pp. 739–794. Edited by B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman & R. E. Shope. New York: Raven Press.

MINOR, P. D. (1980). Comparative biochemical studies of type 3 poliovirus. *Journal of Virology* **34**, 73–84.

MINOR, P. D., SCHILD, G. C., BOOTMAN, J., EVANS, D. M. A., FERGUSON, M., REEVE, P., SPITZ, M., STANWAY, G., CANN, A. J., HAUPTMANN, R., CLARKE, L. D., MOUNTFORD, R. C. & ALMOND, J. W. (1983). Location and primary structure of a major antigenic site for poliovirus neutralization. *Nature, London* **301**, 674–679.

MINOR, P. D., FERGUSON, M., EVANS, D. M. A., ALMOND, J. W. & ICENOGLE, J. P. (1986). Antigenic structure of polioviruses of serotypes 1, 2 and 3. *Journal of General Virology* **67**, 1283–1291.

NEWTON, S. E., CARROLL, A. R., CAMPBELL, R. O., CLARKE, B. E. & ROWLANDS, D. J. (1985). The sequence of foot-and-mouth disease virus RNA to the 5′ side of the poly(C) tract. *Gene* **40**, 331–336.

PALLANSCH, M. A., KEW, O. M., SEMLER, B. L., OMILIANOWSKI, D. R., ANDERSON, C. W., WIMMER, E. & RUECKERT, R. (1984). Protein processing map of poliovirus. *Journal of Virology* **49**, 873–880.

PINCUS, S. E., DIAMOND, D. C., EMINI, E. A. & WIMMER, E. (1986). Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. *Journal of Virology* **57**, 638–646.

RACANIELLO, V. R. & MERIAM, C. (1987). Polio temperature-sensitive mutant containing a single nucleotide deletion in the 5′-noncoding region of the viral RNA. *Virology* (in press).

ROSSMANN, M. G., ARNOLD, E., ERICKSON, J. W., FRANKENBERGER, E. A., GRIFFITH, J. P., HECHT, H. J., JOHNSON, J. E., KAMER, G., LUO, M., MOSSER, A. G., RUECKERT, R. R., SHERRY, B. & VRIEND, G. (1985). The structure of a human common cold virus (rhinovirus 14) and its functional relations to other picornaviruses. *Nature, London* **317**, 145–153.

RUECKERT, R. R. (1985). Picornaviruses and their replication. In *Virology*, pp. 705–738. Edited by B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman & R. E. Shope. New York: Raven Press.

RYAN, M. D. (1985). *Molecular cloning and nucleotide sequence studies on enterovirus 70*. Ph.D. dissertation, University of Leicester.

SARNOW, P., BERNSTEIN, H. D. & BALTIMORE, D. (1986). A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. *Proceedings of the National Academy of Sciences, U.S.A.* **83**, 571–575.

SHERRY, B. & RUECKERT, R. (1985). Evidence for at least two dominant neutralisation antigens on human rhinovirus 14. *Journal of Virology* **53**, 137–143.

SKERN, T., SOMMERGRUBER, W., BLAAS, D., GRÜNDLER, P., FRAUNDORFER, F., PIELER, C., FOGY, I. & KUECHLER, E. (1985). Human rhinovirus 2: complete nucleotide sequence and proteolytic processing signals in the capsid protein region. *Nucleic Acids Research* **13**, 2111–2126.

SKERN, T., NEUBAUER, C., FRASEL, L., GRÜNDLER, P., SOMMERGRUBER, W., ZORN, M., KUECHLER, E. & BLAAS, D. (1987). A neutralizing epitope on human rhinovirus type 2 includes amino acid residues between 153 and 164 of viral capsid protein VP2. *Journal of General Virology* **68**, 315–323.

STADEN, R. (1980). A new computer method for the storage and manipulation of DNA gel reading data. *Nucleic Acids Research* **8**, 3673–3694.

STALHANDSKE, P. O. K., LINDBERG, M. & PETTERSSON, U. (1984). Replicase gene of coxsackievirus B3. *Journal of Virology* **51**, 742–746.

STANWAY, G., CANN, A. J., HAUPTMANN, R., HUGHES, P., CLARKE, L. D., MOUNTFORD, R. C., MINOR, P. D., SCHILD, G. C. & ALMOND, J. W. (1983). The nucleotide sequence of poliovirus type 3 Leon 12 a_1b: comparison with poliovirus type 1. *Nucleic Acids Research* **11**, 5629–5643.

STANWAY, G., HUGHES, P. J., MOUNTFORD, R. C., MINOR, P. D. & ALMOND, J. W. (1984*a*). The complete nucleotide sequence of a common cold virus: human rhinovirus 14. *Nucleic Acids Research* **12**, 7859–7875.

STANWAY, G., HUGHES, P. J., MOUNTFORD, R. C., REEVE, P., MINOR, P. D., SCHILD, G. C. & ALMOND, J. W. (1984*b*). Comparison of the complete nucleotide sequences of the genomes of the neurovirulent poliovirus P3/Leon/37 and its attenuated Sabin vaccine derivative P3/Leon/12a_1b. *Proceedings of the National Academy of Sciences, U.S.A.* **81**, 1539–1543.

STANWAY, G., MOUNTFORD, R. C., COX, S. D. J., SCHILD, G. C., MINOR, P. D. & ALMOND, J. W. (1984*c*). Molecular cloning of the genomes of poliovirus type 3 strains by the cDNA : RNA hybrid method. *Archives of Virology* **81**, 67–78.

TAKEGAMI, T., SEMLER, B. L., ANDERSON, C. W. & WIMMER, E. (1983). Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides. *Virology* **128**, 33–47.

TOYODA, H., KOHARA, M., KATAOKA, Y., SUGANUMA, T., OMATA, T., IMURA, N. & NOMOTO, A. (1984). Complete nucleotide sequences of all three poliovirus serotype genomes. Implication for genetic relationships, gene function and antigenic determinants. *Journal of Molecular Biology* **174**, 561–586.

TOYODA, H., NICKLIN, M. J. H., MURRAY, M. G., ANDERSON, C. W., DUNN, J. J., STUDIER, F. W. & WIMMER, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. *Cell* **45**, 761–770.

TRACY, S., LIU, H. L. & CHAPMAN, N. M. (1985). Coxsackie B3: primary structure of the 5′ non-coding and capsid protein coding regions of the genome. *Virus Research* **3**, 263–270.

YOON, J. W., AUSTIN, M., ONODERA, T. & NOTKINS, A. L. (1979). Virus-induced diabetes mellitus. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *New England Journal of Medicine* **300**, 1173–1179.

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Identification of candidate sequences that determine virulence in Coxsackievirus B4

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Summary

We have previously shown that a major determinant of virulence for coxsackievirus B4 mapped to the 5′ end of the viral genome. Comparison of the corresponding cDNA sequences of a virulent and a non-virulent virus has allowed the identification of candidate determinants of virulence in the 5′ untranslated region and the capsid proteins VP1, VP2 and VP4. Thirteen nucleotide substitutions were observed in a region spanning 3298 nucleotides. Four mutations were detected in the non-coding region. Of the remaining nine mutations, four were silent while five resulted in amino acid substitutions in VP1, VP2 and VP4. The amino acid substitutions in the virulent virus were analyzed in relation to the three-dimensional structures of the capsid proteins of poliovirus. Two substitutions mapped to the amino termini of VP1 and VP4. Of the two substitutions observed in VP2, one mapped to the large loop that connects beta strand E with the radial helix on the back surface of the eight-stranded antiparallel beta barrel while the other mapped to beta strand G. One amino acid substitution in VP1 mapped to the loop connecting beta strands D and E at a site close to a major determinant of attenuation in poliovirus type 2.

Coxsackievirus B4; Virulence; DNA sequence

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A great deal of information exists about the biochemical, biophysical and genetic characteristics of picornaviruses. However, the mechanisms by which these RNA viruses cause disease are poorly understood. Coxsackieviruses of the B group have been implicated in diseases such as pancreatitis, myocarditis, myositis and type I insulin-dependent diabetes mellitus (Grist et al., 1978; Melnick, 1985). Of the group B viruses, variants exist within a single serotype thereby contributing to the variability in the pathogenesis of coxsackievirus infections. We have previously described a variant of coxsackievirus B4 (CB4-V) that induces acute pancreatitis with concurrent hypoglycemia in mice (Ramsingh et al., 1989). The prototypical JVB strain (CB4-P), at similar titers, was non-virulent since infected mice did not develop disease.

A powerful tool in the investigation of the genetic basis of virulence of picornaviruses has been the use of recombinant, chimeric viruses constructed from infectious cDNA clones of virulent and non-virulent viruses (Kohara et al., 1988; Murray et al., 1988). Using this approach, we showed that, for coxsackievirus B4, a major determinant of virulence maps to the 5′ end of the genome, which encompasses the 5′ untranslated region (UTR) and the P1 region, which encodes the four capsid proteins (Ramsingh et al., 1990). Attenuation determinants for poliovirus have also been mapped to this region. Sequence analyses have identified two such determinants in the type 3 vaccine strain, P3/Sabin, and include a uridine at position 472 in the 5′ UTR and a phenylalanine at amino acid 91 of the capsid protein VP3 (Westrop et al., 1987; Minor et al., 1989). Similar studies using the type 1 vaccine strain have identified a strong attenuation determinant in the 5′ UTR, which corresponds to a guanine at position 480, although additional determinants are scattered throughout the genome (Kohara et al., 1985; Nomoto et al., 1987). Recent studies have identified two major determinants of attenuation in type 2 poliovirus (Ren et al., 1991; Equestre et al., 1991). These map to nucleotide 481 in the 5′ UTR and amino acid position 143 of the capsid protein VP1. For another picornavirus, Theiler's murine encephalomyelitis virus (TMEV), amino acid 101 of VP1 has been identified as an important determinant of neurovirulence (Zurbriggen et al., 1991).

In this report, comparison of the sequence data for the 5′ UTR and the P1 regions of a virulent and a non-virulent coxsackievirus B4 has allowed the identification of candidate determinants of virulence in the 5′ UTR and the capsid proteins VP1, VP2 and VP4. The capsid protein VP3 did not appear to contribute to the virulent phenotype. In addition, one amino acid substitution in VP1 mapped to a region very close to a major determinant of attenuation in poliovirus type 2.

The prototypical, non-virulent coxsackievirus B4 (strain JVB), herein designated CB4-P, was kindly provided by R. Deibel (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY). The origin and passage history of a virulent, pancreatropic variant of coxsackievirus B4 (CB4-V) has been previously described (Ramsingh et al., 1989). Viruses were grown in LLC-MK2(D) cells and viral infectivity was determined using a plaque assay or a microtiter $TCID_{50}$ assay (Minor, 1985). Using RNA extracted from purified preparations of either CB4-P or CB4-V, cDNA libraries were prepared as previ-

ously described (Ramsingh et al., 1990). This procedure generated several clones that spanned 95% of the viral genome. To clone the extreme 5′ end of the viral RNA, the technique of polymerase chain reaction (PCR) (Saiki et al., 1986) was used as previously described (Ramsingh et al., 1990). Briefly, oligonucleotide primers derived from the sequence of Jenkins et al. (1987) were used to amplify the 3′ end of the cDNA product that was synthesized by reverse transcription of viral RNA. The cDNA corresponding to bases 1 to 1153 of the viral RNA was amplified. An *Xba*I site was added to the 5′-end primer while an inherent *Eco*RI site was used for the 3′-end primer. The amplified products were subcloned into the phagemid pBSKSM13 + (Stratagene). The sequences of the cDNA inserts were determined by the dideoxy chain-termination method (Sanger et al., 1977) using coxsackievirus-specific primers and Sequenase (U.S. Biochemical). The cDNA inserts were sequenced in both the forward and reverse orientations. Sequence data for the 5′ UTR and the P1 regions of both CB4-V and CB4-P were assembled and analyzed using the Wisconsin GCG Sequence Analysis Software Package (Devereux et al., 1984). Sequence differences between CB4-V and CB4-P were confirmed by RNA sequencing. RNA was sequenced directly by a modification of the dideoxy chain-termination method (Sanger et al., 1977) using virus-specific primers and reverse transcriptase (Life Sciences, St. Petersburg, FL).

Comparison of the cDNA sequence of the 5′ UTR and the P1 region of CB4-V with that of CB4-P revealed a total of 13 mutations, all of which consisted of nucleotide substitutions (Fig. 1). Four substitutions were detected in the 5′ UTR at positions 171, 638, 668 and 683. Of the nine mutations in the coding region for the capsid proteins, four were silent (Table 1). The remaining five mutations occurred in all three codon positions and resulted in amino acid substitutions in the VP1, VP2 and VP4 capsid proteins. Although a point mutation at nucleotide position 2240 in VP3 was noted, this change was silent. The VP1 and VP2 capsid proteins each contained two mutations while the VP4 protein contained one amino acid substitution. In CB4-V, amino acids 20 (Arg^{20}) and 129 (Thr^{129}) of VP1 were different to that of CB4-P (Ser^{20}, Met^{129}). For VP2 of CB4-V, amino acids 135 (Ala^{135}) and 201 (Ala^{201}) were altered (CB4-P, Thr^{135}, Val^{201}). In VP4 of CB4-V, the mutation was at amino acid 16 (Arg^{16}) (CB4-P, Ser^{16}). Of the amino acid substitutions, Ala^{201} of VP2 (CB4-V) represented a conservative change while the remaining substitutions resulted in alterations in hydrophobicity or charge. Ala^{135} of VP2 (CB4-V) is more hydrophobic while Thr^{129} of VP1 (CB4-V) is more hydrophilic than the corresponding residues in CB4-P. Two mutations, Arg^{16} and Arg^{20} at the amino termini of VP4 and VP1, respectively, resulted in charge differences.

Comparison of our sequence data for CB4-P (strain JVB) with those of another sequence of strain JVB by Jenkins et al. (1987), herein referred to as CB4*, revealed a total of nine nucleotide substitutions between these two viruses (Fig. 1). The mutations probably reflect the different passage histories of the two viruses. Four of the substitutions mapped to the 5′ UTR at nucleotide positions 136, 137, 546 and 668. Of the five remaining mutations, two were silent and three resulted in amino acid substitutions (Table 1). These three changes mapped to the capsid

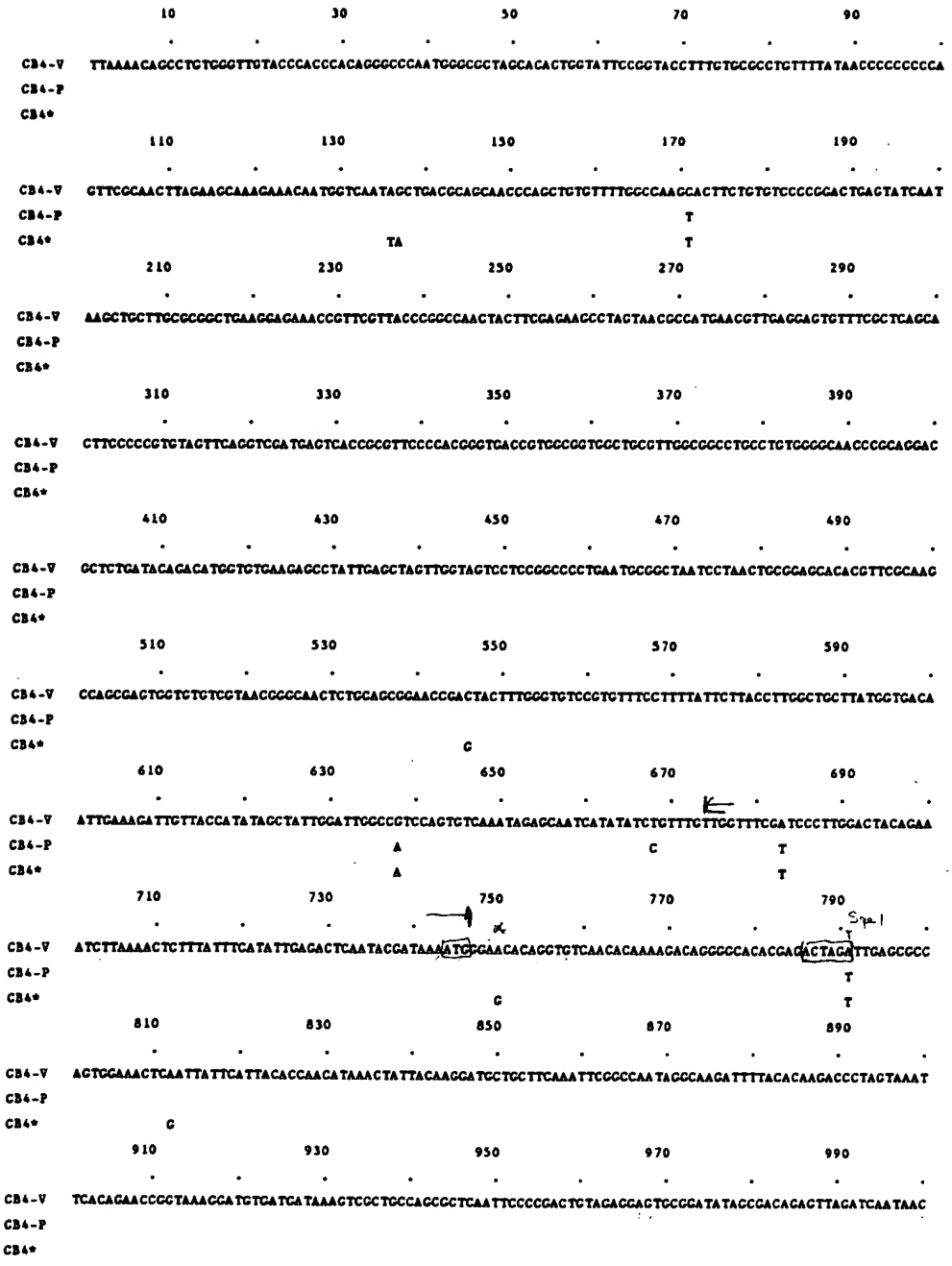

Fig. 1. (pp 284–287). Comparison of the cDNA sequences of the 5' untranslated region (nucleotides 1–743) and the P1 region (nucleotides 744–3298) of CB4-V, CB4-P and CB4* (Jenkins et al., 1987).

```
                1010                1030                1050                1070                1090
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   ACTCGGGAACTCGACTATAACGACACAAGAGTGTGCAAACGTCGTGGTGGGGTATGGCGTCTGGCCCGATTATCTTAGCGACGAAGAGGCAACAGCGGAA
CB4-P
CB4*

                1110                1130                1150                1170                1190
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   GACCAACCCACCCAACCTCATGTGGCAACGTGTAGGTTTTACACGTTGAATTCAGTGAAATGGGACATGCAGTCAGCGGGGTGGTGGTGAAGTTCCCAG
CB4-P   G
CB4*    G
                1210                1230                1250                1270                1290
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   ATGCATTGTCAGAAATGGGGCTCTTTGGGCAGAATATGCAGTATCACTACCTAGGCAGATCAGGGTACAGAATTCATGTGCAATGCAACGCATCCAAATT
CB4-P                                   A
CB4*                                    A
                1310                1330                1350                1370                1390
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   CCACCAAGGTTGTCTGCTTGTGGTGTGTGTGCCTGAGGCTGAGATGGGATGTGCCAATGCAGAAAACGCACCCGCGTATGGTGATTTGTGTGGAGGAGAG
CB4-P                                           A
CB4*                                            A
                1410                1430                1450                1470                1490
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   ACAGCAAAGAGTTTCGAACAGAATGCAGCCACAGGTGAGACAGCTGTGCAGACGGCTGTGTGCAATGCGGGTATGCGTGTGCGGGGTTGGTAACTTGACTA
CB4-P
CB4*                                    A
                1510                1530                1550                1570                1590
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   TATACCCTCACCAATGGATTAATTTAAGAACAAACAATAGTGCCACCATAGCGATGCCATACATTAATAGCGTCCCAATGGACAACATGTTCAGGCATAA
CB4-P                                           T
CB4*                                            T
                1610                1630                1650                1670                1690
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   TAACTTTACATTAATGATAATACCCTTTGCACCGTTGGACTACGTTACGGGAGCGTCCTCTTACATCCCTATCACAGTGACAGTTGCCCCTATGAGCGCT
CB4-P
CB4*
                1710                1730                1750                1770                1790
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   GAGTACAATGGTTTGCGTCTAGCTGGTCATCAAGGCTTACCAACTATGCTTACACCAGGCAGCACGCAGTTTTTGACGTCAGATGATTTTCAATCACCAT
CB4-P
CB4*
                1810                1830                1850                1870                1890
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   CAGCTATGCCACAGTTTGATGTGACCCCAGAGATGAACATTCCAGGGCAAGTGAGGAACCTGATGGAAATTGCGGAAGTTGATTCTGTGGTACCAATCAA
CB4-P
CB4*
                1910                1930                1950                1970                1990
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   TAACTTGAAAGCTAATCTGATGACGATGGAGGCTTACCGGGTGCAGGTTAGGTCCACTGACGAGATGGGAGGACAGATATTTGGCTTCCCCTTACAGCCA
CB4-P
CB4*
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Fig. 1 (continued).

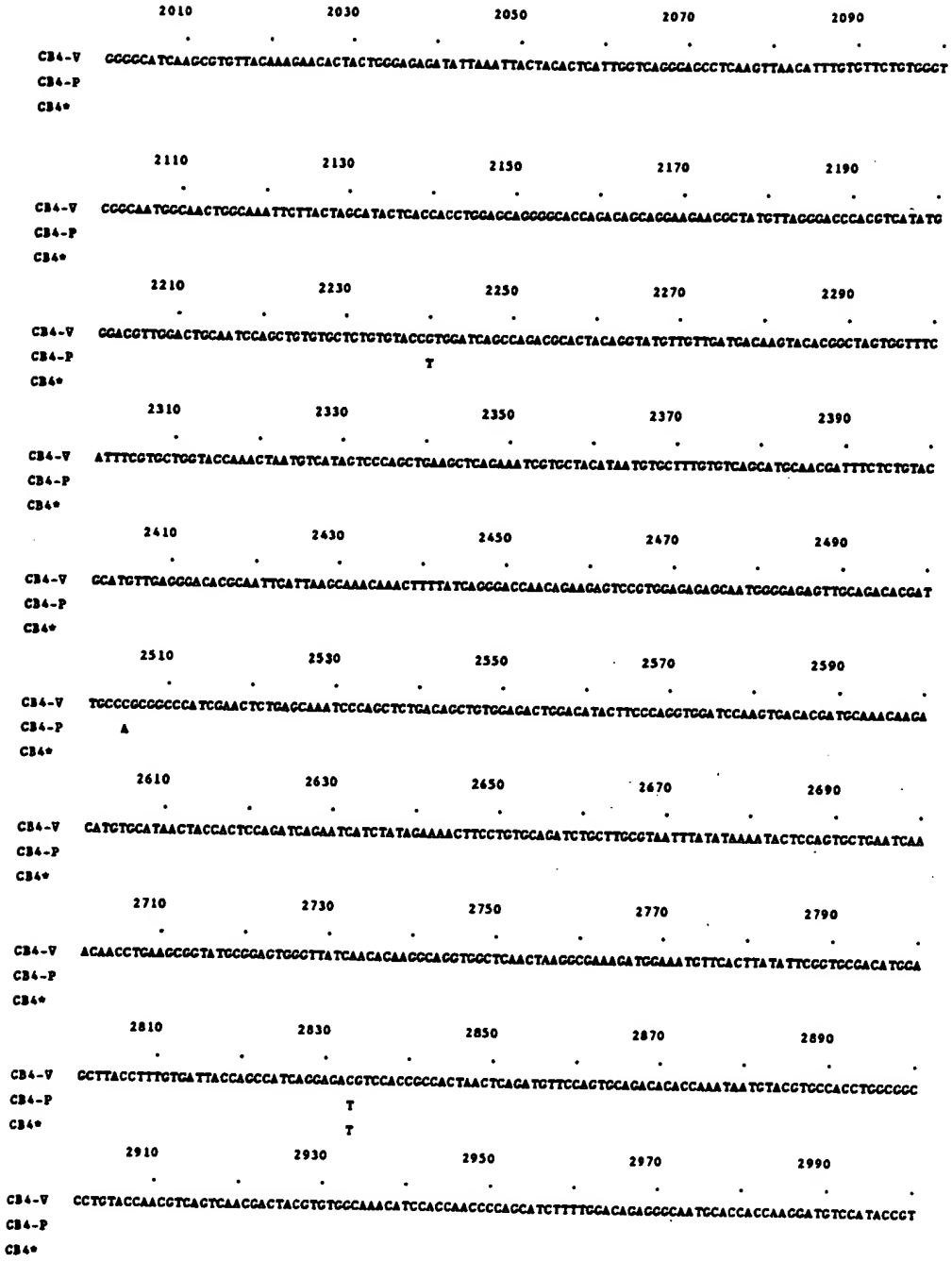


Fig. 1 (continued).

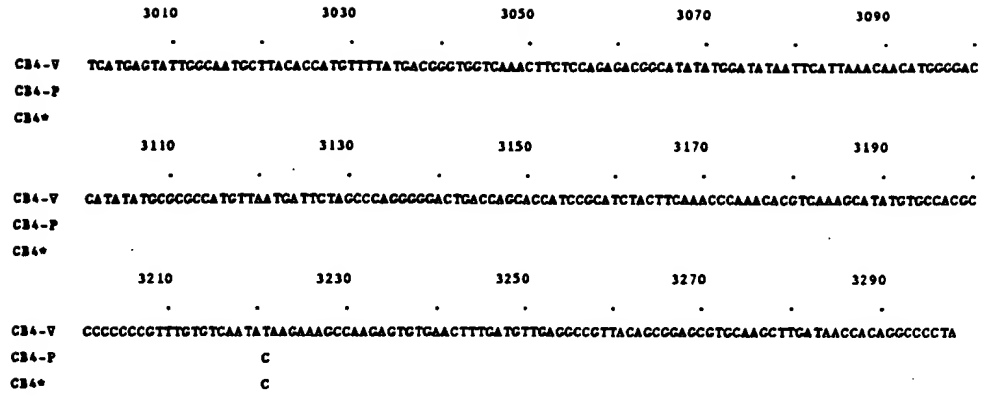


Fig. 1 (continued).

proteins VP4, VP2 and VP1. Again, although a point mutation was observed in VP3 at nucleotide position 2240, this change was silent. CB4-P contained Thr^{3} (VP4), Glu^{163} (VP2) and Ser^{20} (VP1) while the JVB strain sequenced by Jenkins et al. (1987) had Ala^{3} (VP4), Lys^{163} (VP2) and Arg^{20} (VP1).

TABLE 1

Summary of sequence data for the 5'UTR and the P1 regions of CB4-V, CB4-P and CB4*

Sequenced nucleotide	Genome region	Nucleotide				Amino acid			
		Position	CB4-P	CB4-V	CB4*	Position	CB4-P	CB4-V	CB4*
1-743	5'UTR	136	A	A	T				
		137	G	G	A				
		171 [a]	T	C	T				
		546	C	C	G				
		638 [a]	A	G	A				
		668 [b]	C	T	T				
		683 [a]	T	A	T				
744-949	VP4	750	A	A	G	3	Thr	Thr	Ala
		791 [a]	T	A	T	16	Ser	Arg	Ser
		812	A	A	G	23	Ser	Ser	Ser
950-1732	VP2	1106	G	A	G	52	Gly	Gly	Gly
		1241	A	G	A	97	Gly	Gly	Gly
		1353 [a]	A	G	A	135	Thr	Ala	Thr
		1437	G	G	A	163	Glu	Glu	Lys
		1552 [a]	T	C	T	201	Val	Ala	Val
1733-2446	VP3	2240	T	G	G	169	Pro	Pro	Pro
2447-3298	VP1	2505 [b]	A	C	C	20	Ser	Arg	Arg
		2833 [a]	T	C	T	129	Met	Thr	Met
		3221	C	T	C	258	Tyr	Tyr	Tyr

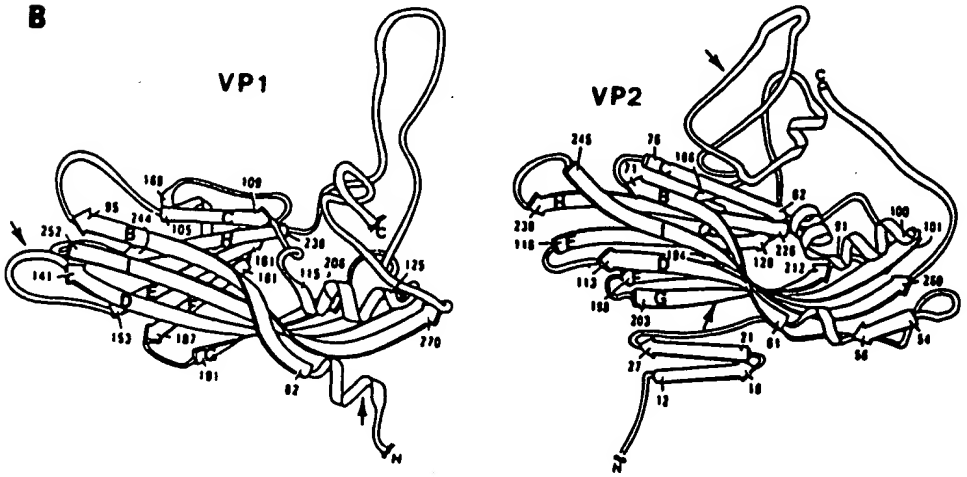
[a] Potential determinant of virulence.
[b] Common nucleotide between CB4-V and CB4*.

A

```
                1                                                  *      50
CB4-V VP1   .......GPT EESVERAMGR VADT...... IARGPSNSEQ IPALTAVETG
Polio VP1   GIGDMIEGAV EGITKNALVP PTSTNSLPGH KPSGPAHSKE IPALTAVETG
               51                                                     100
CB4-V VP1   HTSQVDPSDT MQTRHVHNYH SRSESSIENF LCRSACV..I YIKYSSAESN
Polio VP1   ATNPLVPSDT VQTRHVIQRR TRSESTVESF FARGACVAII EVDNDAPTKR
              101                                                  *  150
CB4-V VP1   NLKRYAEWVI NTRQVAQLRR KMEMFTYIRC DMELTFVITS HQETSTATNS
Polio VP1   ASRLFSVWKI TYKDTVQLRR KLEFFTYSRF DMEFTFVVTS ..NYIDANNG
              151                                                  ▲  200
CB4-V VP1   DVPVQTHQIM YVPPGGPVPT SVNDYVWQTS TNPSIFWTEG NAPPRMSIPF
Polio VP1   HALNQVYQIM YIPPGAPIPG KWNDYTWQTS SNPSVFYTYG APPARISVPY
              201                                                    250
CB4-V VP1   MSIGNAYTMF YDGWSNFS.. ......RDGI YGYNSLNNMG TIYARHVNDS
Polio VP1   VGIANAYSHF YDGFAKVPLA GQASTEGDSL YGAASLNDFG SLAVRVVNDH
              251                                                    300
CB4-V VP1   SPGGLTSTIR IYFKPKHVKA YVPRPPRLCQ YKKAKSVNFD VEAVTAERAS
Polio VP1   NPTRLTSKIR VYMKPKHVRV WCPRPPRAVP Y.FGPGVDYK .DGLTPLPEK
              301
CB4-V VP1   LITTGPY
Polio VP1   GLTTY..
```

```
                1                                                      50
CB4-V VP2   SPTVEECGYS DRVRSITLGN STITTQECAN VVVGYGVWPD YLSDEEATAE
Polio VP2   SPNIEACGYS DRVMQLTLGN STITTQEAAN SVVAYGRWPE YIRDTEANPV
               51                                                     100
CB4-V VP2   DQPTQPDVAT CRFYTLNSVK WEMQSAGWWW KFPDALSEMG LFGQNMQYHY
Polio VP2   DQPTEPDVAA CRFYTLDTVT WRKESRGWWW KLPDALKDMG LFGQNMFYHY
              101                                                  *  150
CB4-V VP2   LGRSGYTIHV QCNASKFHQG CLLVVCVPEA EMG.......  ...CANAENA
Polio VP2   LGRAGYTVHV QCNAFKFHQG ALGVFAVPEM CLAGDSTTHM FTKYENANPG
              151                                                    200
CB4-V VP2   PAYGDLCGGE TAKSFEQNAA TGETAVQTAV CNAGMGVGVG NLTIYPHQWI
Polio VP2   EKGGEFKGSF T...LDTNAT NPARNFCPVD YLFGSGVLVG NAFVYPHQII
              201                *                                   250
CB4-V VP2   NLRTNNSATI AMPYINSVPM DNMFRHNNFT LMIIPFAPLD YVTGASSYIP
Polio VP2   NLRTNNCATL VLPYVNSLSI DSMTKHNNWG IAILPLAPLD FVTESSTEIP
              251                                273
CB4-V VP2   ITVTVAPMSA EYNGLRLAGH Q..
Polio VP2   ITLTIAPMCC EFNGLRNITV PRT
```

B



There is a high degree of similarity between the predicted amino acid sequences of coxsackieviruses and polioviruses. Comparison of the amino acid sequences of VP1 and VP2 of CB4-V to that of poliovirus type 2 (P712) (Toyoda et al., 1984) revealed sequence identities of 43.6% and 54.9%, respectively. Therefore, the amino acid changes observed in CB4-V were analyzed in relation to the three-dimensional structures of the capsid proteins of poliovirus. Alignment of the amino acid sequences of VP1 and VP2 of CB4-V with the corresponding capsid proteins of poliovirus type 2 (P712) was accomplished with the GCG program PILEUP (Fig. 2A). Using data from the three-dimensional structure of poliovirus type 1 (Mahoney strain) (Hogle et al., 1985) (permission kindly granted by J. Hogle and the AAAS, copyright 19 by the AAAS), this alignment allowed an approximation of the region of the molecule where the substituted amino acids in CB4-V mapped (Fig. 2B). By this comparison, Thr^{129} of VP1 would map to the loop connecting beta strands D and E while Arg^{20} would map to the amino terminus. Ala^{135} of VP2 would map to the large loop that connects beta strand E with the radial helix on the back surface of the eight-stranded antiparallel beta barrel while Ala^{201} would map to beta strand G. From this alignment, we can predict that Thr^{129} (VP1) and Ala^{135} (VP2) would be on the surface of the virion while Arg^{20} (VP1), Ala^{201} (VP2) and Arg^{16} (VP4) would be expected to reside in the interior of the virion.

Comparison of the cDNA sequences of a virulent coxsackievirus B4 (CB4-V) and a non-virulent virus (CB4-P) revealed a total of thirteen point mutations. Four mutations occurred in the 5′ UTR while nine changes were observed in the P1 region. Of the nine mutations, five resulted in amino acid substitutions while four were silent. Comparison of the cDNA sequences of the 5′ UTR and the P1 regions of the two JVB strains of coxsackievirus B4 (CB4-P and CB4*) revealed nine nucleotide substitutions, which probably reflect the different passage histories of the two viruses. Four nucleotide changes were observed in the 5′ UTR while five substitutions were seen in the P1 region. Of the five changes, two were silent while three resulted in amino acid substitutions. This analysis suggests that CB4-V and CB4-P are just as similar as the two JVB strains, CB4-P and CB4*. All of the amino acid differences observed in the P1 region of the various strains occurred in the capsid proteins VP1, VP2 and VP4.

Of the thirteen point mutations observed between CB4-V and CB4-P, three (nucleotide positions 668, 2240 and 2505) were shared between the two JVB strains (Table 1). Since the point mutation at position 2240 is silent, these data suggest that the candidate determinants of virulence for CB4-V may be narrowed further to include three nucleotides in the 5′ UTR (171, 638, 683), Arg^{16} of VP4, Ala^{135}

Fig. 2. Alignment of the capsid proteins VP1 and VP2 of CB4-V to those of poliovirus. (A) Alignment of the amino acid sequences of VP1 and VP2 of CB4-V with the corresponding capsid proteins of poliovirus type 2 (Toyoda et al., 1984) using the GCG program PILEUP. (B) Ribbon diagrams of VP1 and VP2 of poliovirus type 1 (Hogle et al., 1985) (permission granted by J. Hogle and the AAAS, copyright 19 by the AAAS). *, potential determinant of virulence in CB4-V; arrowhead, determinant of attenuation in poliovirus type 2 (Ren et al., 1991; Equestre et al., 1991); arrows, approximation of sites that potentially contribute to virulence in CB4-V.

and Ala^{201} of VP2 and Thr^{129} of VP1. The capsid protein VP3 apparently does not contribute to virulence in coxsackievirus B4 in our system.

Alignment of the amino acid sequences of VP1 and VP2 of CB4-V with that of poliovirus type 2 (P712) allowed an approximation of the regions of the molecules where the mutations in CB4-V occurred. Interestingly, amino acid 129 (Thr^{129}) of VP1 of CB4-V aligned with amino acid 142 (Tyr^{142}) of VP1 of poliovirus. The alignment positions Thr^{129} on the loop connecting beta strands D and E. In relation to the canyon structure (Rossmann, 1989), the alignment of Thr^{129} places this residue on the rim of the canyon. Recently, it has been shown that amino acid 143 in VP1 of poliovirus type 2 is a major determinant of attenuation (Ren et al., 1991; Equestre et al., 1991). Thus, one of the mutations observed in CB4-V occurred at a position very close to this site in poliovirus. The DE loop is exposed on the external surface of the virion and, in poliovirus, influences both host range and immunogenicity (Ren et al., 1991; Wieggers et al., 1989).

Two mutations in CB4-V, Arg^{20} and Arg^{16} occurred at the amino termini of VP1 and VP4, respectively. These two mutations are expected to be in the interior of the virion. The gain of positive charges that they introduce may affect the interactions of VP1 and VP4 with the negatively-charged viral genomic RNA. For VP2 of CB4-V, a mutation (Ala^{201}) was observed in beta strand G, which is also in the interior of the virion. However, Ala^{201} represents a conservative amino acid change and is not expected to affect virulence. The second, non-silent mutation in VP2 of CB4-V (Ala^{135}) occurred in the large loop that connects beta strand E with the radial helix on the back surface of the barrel and again, this region is predicted to be exposed on the surface of the virion, on the rim of the canyon. Of the three amino acid substitutions observed between the two JVB strains, one also maps to the large loop in VP2 while the remaining two map towards the amino termini of VP1 and VP4.

Of the four point mutations in the UTR, which comprises a total of 743 nucleotides, three were clustered towards the 3′ end of this region (positions 638, 668 and 683) while one mutation occurred towards the 5′ end (position 171). Attenuation determinants have been mapped to the 5′ UTR in all three serotypes of poliovirus (Equestre et al., 1991; Kohara et al., 1985; Nomoto et al., 1987; Ren et al., 1991; Westrop et al., 1987). These determinants cluster in the middle of the 5′ UTR and include nucleotide positions 480, 481 and 472 of poliovirus types 1, 2 and 3 respectively. Thus, the four point mutations in the 5′ UTR of CB4-V occur at sites different to those observed for polioviruses.

For coxsackievirus B4, candidate determinants of virulence have been localized to the 5′ UTR and the capsid proteins VP1, VP2 and VP4. For the polioviruses, determinants of attenuation have been mapped to the 5′ UTR and the capsid proteins VP1 and VP3 (Equestre et al., 1991; Kohara et al., 1985; Minor et al., 1989; Nomoto et al., 1987; Ren et al., 1991; Westrop et al., 1987). Of the nine potential sites in CB4-V, one (Thr^{129}) maps to the DE loop of VP1, at a position close to a major determinant of attenuation in poliovirus type 2 (Ile^{143}). The remaining eight sites in CB4-V do not coincide with any other determinants in poliovirus. Should Thr^{129} of VP1 be a determinant of virulence in CB4-V, then the

molecular mechanisms underlying virulence in coxsackieviruses and polioviruses may share some common features.

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References

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12, 387–395.

Equestre, M., Genovese, D., Cavalieri, F., Fiore, L., Santoro, R. and Bercoff, R.P. (1991) Identification of a consistent pattern of mutations in neurovirulent variants derived from the Sabin vaccine strain of poliovirus type 2. J. Virol. 65, 2707–2710.

Grist, N.R., Bell, E.J. and Assaad, F. (1978) Enteroviruses in human disease. Prog. Med. Virol. 24, 114–157.

Hogle, J.M., Chow, M. and Filman, D.J. (1985) Three-dimensional structure of poliovirus at 2.9 Å resolution. Science 229, 1358–1365.

Jenkins, O., Booth, J., Minor, P. and Almond, J. (1987) The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the Picornaviridae. J. Gen. Virol. 68, 1835–1848.

Kohara, M., Omata, T., Kameda, A., Semler, B.L., Itoh, H., Wimmer, E. and Nomoto, A. (1985) In vitro phenotypic markers of a poliovirus recombinant constructed from infectious cDNA clones of the neurovirulent Mahoney strain and the attenuated Sabin 1 strain. J. Virol. 53, 786–792.

Kohara, M., Shinobu, A., Komatsu, T., Tago, K., Arita, M., and Nomoto, A. (1988) A recombinant virus between the Sabin 1 and Sabin 3 vaccine strains for poliovirus as a possible candidate for a new type 3 poliovirus live vaccine strain. J. Virol. 62, 2828–2835.

Melnick, J.L. (1985) Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: B.N. Fields, D.M. Knipe, R.M. Chanock, J.L. Melnick, B. Roizman and R.E. Shope (Eds.), Virology, pp. 739–794,Raven, New York.

Minor, P.D. (1985) Growth, assay and purification of picornaviruses. In B.W.J. Mahy (Ed.) Virology, A Practical Approach, pp. 25–41. IRL Press, Oxford, U.K.

Minor, P.D., Dunn, G., Evans, D.M.A., Magrath, D.I., John, A., Howlett, J., Phillips, A., Westrop, G., Wareham, K., Almond, J.W. and Hogle, J.M. (1989) The temperature sensitivity of the Sabin type 3 vaccine strain of poliovirus: molecular and structural effects of a mutation in the capsid protein VP3. J. Gen. Virol. 70, 1117–1125.

Murray, M., Kuhn, R., Arita, M., Kawamura, N., Nomoto, A. and Wimmer, E. (1988) Poliovirus type 1/type 3 antigenic hybrid virus constructed in vitro elicits type 1 and type 3 neutralizing antibodies in rabbits and monkeys. Proc. Natl. Acad. Sci. USA 85, 3203–3207.

Nomoto, A., Kohara, M., Kuge, S., Kawamura, N., Arita, M., Komatsu, T., Abe, S., Semler, B.L., Wimmer, E. and Itoh, H. (1987) Study on virulence of poliovirus type 1 using in vitro modified viruses. In M.A. Brinton and R.R. Rueckert (Eds.), Positive Strand RNA Viruses. pp. 437–452, Liss, New York.

Ramsingh, A., Hixson, A., Duceman, B. and Slack, J. (1990) Evidence suggesting that virulence maps to the P1 region of the Coxsackievirus B4 genome. J. Virol. 64, 3078–3081.

Ramsingh, A., Slack, J., Silkworth, J. and Hixson, A. (1989) Severity of disease induced by a pancreatropic Coxsackie B4 virus correlates with the H-2K^q locus of the major histocompatibility complex. Virus Res. 14, 347–358.

Ren, R., Moss, E.G. and Racaniello, V.R. (1991) Identification of two determinants that attenuate vaccine related type 2 poliovirus. J. Virol. 65, 1377–1382.

Rossmann, M.G. (1989) The canyon hypothesis. Hiding the host cell receptor attachment site on a viral surface from immune surveillance. J. Biol. Chem. 264, 14587–14590.

Saiki, R., Bugawan, T., Horn, G., Mullis, K. and Erlich, H. (1986) Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. Nature 324, 163–166.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Toyoda, H., Kohara, M., Kataoka, Y., Suganuma, T., Omata, T., Imura, N. and Nomoto, A. (1984) Complete nucleotide sequences of all three poliovirus serotype genomes. Implications for genetic relationships, gene function and antigenic determinants. J. Mol. Biol. 174, 561–585.

Westrop, G.D., Evans, D.M.A., Minor, P.D., Magrath, D., Schild, G.C. and Almond, J.W. (1987) Investigation of the molecular basis of attenuation in the Sabin type 3 vaccine using novel recombinant polioviruses constructed from infectious cDNA. In D.J. Rowlands, M.A. Mayo, and B.W.J. Mahy (Eds.), The Molecular Biology of the Positive Strand RNA Viruses. pp. 53–60, Liss, New York.

Wiegers, K., Uhlig, H. and Dernick, R. (1989) NAg1B of poliovirus type 1: a discontinuous epitope formed by two loops of VP1 comprising residues 96–104 and 141–152. Virology 170, 583–586.

Zurbriggen, A., Thomas, C., Yamada, M., Roos, R. and Fujinami, R. (1991) Direct evidence of a role for amino acid 101 of VP-1 in central nervous system disease in Theiler's murine encephalomyelitis virus infection. J. Virol. 65, 1929–1937.

Virus Research, 14 (1989) 347–358
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Severity of disease induced by a pancreatropic Coxsackie B4 virus correlates with the H-2K^q locus of the major histocompatibility complex

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Summary

Coxsackie B viruses are known etiological agents of pancreatic diseases, including diabetes. The pathogenesis of these infections is influenced by both host and viral factors. In this report, we examined whether the outcome of Coxsackie B4 virus infection is dependent on the genes within the major histocompatibility complex (MHC). We generated a pancreatic variant, CB4-V and established an animal model system of pancreatitis with concurrent hypoglycemia in mice. Infection of various B10 H-2 congenic strains of mice revealed that the development of hypoglycemia with accompanying pancreatitis was independent of the MHC haplotype. However, the severity of the disease as monitored by the extent and duration of hypoglycemia and by mortality rate was found to be associated with the H-2 haplotype, specifically the H–2K^q locus. Pancreatic damage induced by CB4-V appeared to be both immune-mediated and viral-mediated. Histological examination of pancreatic tissue from infected B10 H-2 congenic mice revealed an association between acute destruction of the exocrine pancreas and lymphocytic infiltration. This infiltration may correlate with immune-mediated destruction of the infected pancreatic tissue. Since preferential replication of CB4-V was not observed in the most susceptible B10 mouse strain, direct viral destruction may not be the major mechanism of pancreatic injury.

Coxsackie B4; Major histocompatibility complex

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Introduction

The association between Coxsackie viruses of the B group and pancreatic diseases including diabetes has been recognized for many years (Dalldorf and Gifford, 1952; Gamble et al., 1969; Pappenheimer et al., 1951). Initial studies revealed that several Coxsackie B viruses caused extensive destruction of pancreatic acinar tissue in mice without identifiable changes in the cells of the islets of Langerhans (Dalldorf and Gifford, 1952; Pappenheimer et al., 1951). Ross et al. (1974) correlated pathological changes produced in the mouse pancreas by Coxsackie B (CB) viruses with serum amylase and glucose concentrations. They found that, although CB4 infections were less severe than those of CB1, CB3 and CB5, CB4 induced an acinar pancreatitis with a transient increase in serum amylase and a transient decrease in glucose levels. These results contrasted with those of Lansdowne (1976) who showed that the pathogenicity of CB4 closely resembled that seen in animals infected with CB3 and was more severe than that associated with CB1 and CB5 infections. In neither study were visible changes in the islets of Langerhans observed. Subsequent studies by Yoon et al. (1978a, b) and Tóniolo et al. (1982) showed that Coxsackie B viruses could infect pancreatic β cells. Furthermore, inoculation of mice with CB4 virus that had been passaged in mouse β cell cultures resulted in the development of diabetes. Yoon et al. (1978b) suggested that most strains of Coxsackie B virus are minimally β cell-tropic and that if β cells are damaged, the number is usually insufficient to produce detectable alterations in glucose metabolism. However, the tropism of these viruses for insulin-producing cells can be increased by serial passage in β cell cultures. It appears that diabetogenic variants of Coxsackie B virus exist in nature since Yoon et al. (1979) isolated a diabetogenic variant of CB4 from a patient with diabetic ketoacidosis. Thus, the different diseases produced by Coxsackie B viruses may be due to variants with different tropisms and biologic properties within a mixed viral population.

Genetic factors appear to play a role in determining susceptibility to viral infections. Early studies showed that development of encephalomyocarditis (EMC) virus-induced diabetes in various inbred strains of mice is genetically determined and that susceptibility is inherited as a recessive trait (Ross et al., 1976; Yoon and Notkins, 1976). Analysis of the F2 data suggested that more than one gene was involved (Ross et al., 1976). Later studies from backcross data implied that susceptibility to EMC-induced diabetes was primarily controlled by a single locus involving two or more alleles (Onodera et al., 1978). Further work has suggested that genetically determined differences in viral receptors on the surface of pancreatic β cells may be one of the factors controlling susceptibility to EMC-induced diabetes. Susceptibility to CB4-induced diabetes is also genetically determined (Yoon et al., 1978b). As is the case in EMC-induced diabetes, only certain inbred strains of mice develop diabetes when injected with CB4 virus. Most of the strains susceptible to EMC-induced diabetes are also susceptible to CB4-induced diabetes. However, the role of the major histocompatibility complex (MHC) in EMC or CB4 viral infections has not been defined. The present work was undertaken to examine the pathogenesis of CB4 virus infections and to specifically address whether

susceptibility to a pancreatropic variant of CB4 virus was influenced by the genes within the MHC. Since CB4 virus normally infects the acinar cells of the exocrine pancreas, these studies were carried out using a pancreatic variant, CB4-V, which caused acinar pancreatitis with concurrent hypoglycemia in mice.

Materials and Methods

Virus and cells

Coxsackie B4 (JVB) was kindly provided by R. Deibel (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY). A Coxsackie B4 isolate that was originally diabetogenic and that was subsequently grown in LLC-MK2(D) cells was provided by J.W. Yoon (University of Calgary, Alberta, Canada). Large-scale stock preparations of these viruses were prepared in either HeLa or LLC-MK2(D) cells. Viral infectivity was determined using a microtiter $TCID_{50}$ assay (Minor, 1985).

Mice

SJL mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Breeders of strains B10.Q, B10.S(12 R) and B10.T(6R) and B10.AQR and B10.AKM were provided by J. Stimpfling (The McLaughlin Research Institute, Great Falls, Montana) and K. Frederick (Washington University, St. Louis, MO), respectively. All mice used in these experiments were 4–6-week-old males and were maintained 3 per cage. Mice were allowed to eat and drink ad libitum. Mice were injected intraperitoneally (IP) with virus diluted in phosphate-buffered saline (PBS). Control mice were injected IP with PBS. All injected animals were monitored daily. Animals found to be moribund were euthanized immediately by CO_2 overdose and counted as fatalities. All animal procedures were in accord with Department of Health, Education, and Welfare (DHEW) Publ. No. (NIH) 86–23, "Guide for the Care and Use of Laboratory Animals", and had been reviewed and approved by the Wadsworth Center Animal Welfare Committee.

Glucose assay

Non-fasted mice were bled from the tail vein and serum glucose concentrations were determined by the glucose oxidase method (Raabo and Terkildsen, 1960). For glucose tolerance tests, blood samples were collected before and 60 min after intraperitoneal administration of 2 mg of D-glucose per gram body weight. Mice were fasted during the 60 min glucose challenge.

Histopathology

At autopsy, the pancreas was removed aseptically and rinsed in sterile PBS. One half of the pancreas was fixed in phosphate-buffered formalin and sections were

stained by hematoxylin and eosin. The other half was processed for the presence of infectious virus. These mice were euthanized by ether overdose.

Extraction of virus from various organs

Pancreas, heart, kidney and spleen were removed aseptically and rinsed in sterile PBS. After mincing in PBS, cells were broken by homogenization in a Dounce tissue grinder. Cell-associated virus was released after 3 cycles of freezing and thawing. After a clarifying spin at $5000 \times g$ for 10 min to remove cellular debris, the supernate was collected and filtered through a 0.2 μM filter. Homogenates were tested for viral infectivity using a microtiter $TCID_{50}$ assay (Minor, 1985).

Results

Coxsackie B4 virus variant induces severe and prolonged hypoglycemia in SJL mice

The Coxsackie B4 virus, obtained from Dr. Yoon (University of Calgary, Alberta, Canada), had initially been passaged 15 times in human pancreatic β cells and was diabetogenic in susceptible mouse strains. Subsequent passages had been carried out in LLC-MK2(D) cells. To test whether this virus had retained its diabetogenic potential, each of twenty 4–6-week-old male SJL mice was injected intraperitoneally (IP) with $10^{6.3}$ $TCID_{50}$ of virus and serum glucose concentrations were monitored at various times post-infection (PI). These animals remained normoglycemic during the 8 weeks PI (data not shown) suggesting that the diabetogenic variants within this mixed viral population were lost by passaging in LLC-MK2(D) cells. To enhance the pancreatropic nature of this virus, it was then passaged in mice (via pancreatic homogenates) (Ross et al., 1974) a total of 5 times. The resulting virus has been designated CB4-variant (CB4-V).

To determine whether CB4-V differed from the prototypical CB4 (JVB) herein designated (CB4-P) virus, SJL mice were injected IP with $10^{4.3}$ $TCID_{50}$ of virus or 0.2 ml PBS and serum glucose concentrations were determined at various times PI (Fig. 1). Mice infected with CB4-P had serum glucose levels similar to that of PBS-inoculated control mice. However, all mice infected with CB4-V developed severe hypoglycemia (serum glucose 30 mg/dl $\pm$ 2 1 as compared to 131 $\pm$ 14 for PBS-injected controls) within 4 days PI. Fifty percent of these animals became moribund and were euthanized within 7 days PI. The remaining animals exhibited a prolonged hypoglycemia. Serum glucose levels of these mice were more than 3 standard deviations below normal for 5 weeks PI and returned to normal at 8 weeks PI. During the acute phase which lasted 2 weeks, infected mice had distended abdomens and appeared huddled, inactive and weighed 25–30% less than control animals. To detect additional abnormalities in glucose metabolism after infection with CB4-V, glucose tolerance tests were performed 7 days PI. Infected mice cleared the glucose load efficiently (data not shown). Thus, the hypoglycemic animals did not show abnormal glucose tolerance.

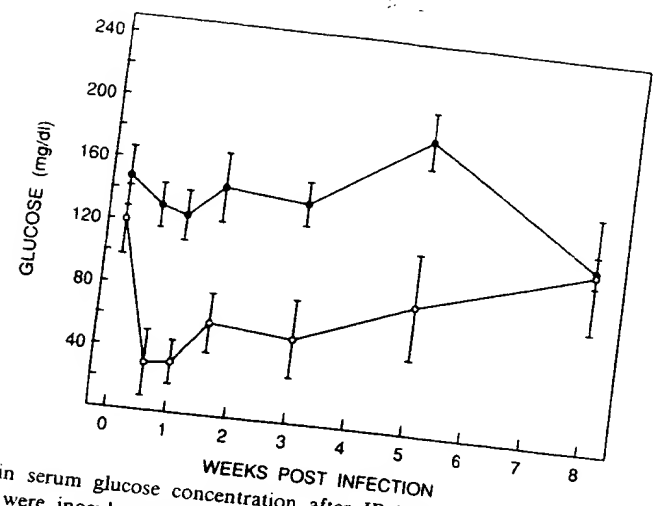

Fig. 1. Reduction in serum glucose concentration after IP inoculation with CB4-V. Groups of 30 4–6-week-old mice were inoculated with $10^{4.3}$ $TCID_{50}$ of CB4-V (○——○) and serum glucose concentrations were monitored for 8 weeks post infection. Fifty percent of these animals were moribund within 7 days PI. Groups of 5–10 4–6-week-old mice were inoculated with PBS (●——●) and serum glucose concentrations were again monitored for 8 weeks. No deaths occurred in this group.

The intensity and duration of the hypoglycemia observed in CB4-V-infected SJL mice could be attenuated by decreasing the virus inoculum. Infection with $10^{2.7}$ $TCID_{50}$ of CB4-V resulted in a less severe and prolonged hypoglycemia (Table 1). The mortality rate for these animals was 0%.

TABLE 1

Susceptibility of various mouse strains to CB4-V

Mouse strain	H-2 haplotype			Mortality rate [a] (%)	Duration of hypoglycemia [b] (weeks)	Severity of symptoms at 7–14 days PI [c]
	K	I	D			
B10.T(6R)	q	q	d	100	2	+ + + + [d]
B10.Q	q	q	q	43–69	6–7	+ + +
B10.AQR	q	k	d	45–60	6–7	+ + +
B10.AKM	k	k	q	0	3–4	+
B10.S(12R)	s	s	s	0	3–4	+
SJL	s	s	s	0	2–3	+

[a,b,c] Summary of results obtained from 3 independent experiments. The shortened duration of hypoglycemia observed in infected B10.T(6R) mice reflects the fact that these animals were moribund within a 2-week period.

[d] Symptoms were monitored daily and graded from mild (+) to extremely severe (+ + + +). +: huddled, active; + + +: huddled, inactive, weight loss; + + + +: huddled, inactive, shivering, weight loss (these animals were defined as moribund).

CB4-V causes severe acinar pancreatitis in SJL mice

To correlate histological changes in the pancreas with CB4-V-induced hypoglycemia, tissue from animals infected with $10^{4.3}$ $TCID_{50}$ of virus, was harvested at various times PI and processed for staining by hematoxylin and eosin (Fig. 2A–C). At 4 days PI, degeneration of the exocrine pancreas was observed in SJL mice as a generalized degranulation of the acinar cells and partial loss of the number of exocrine secretory units. Upon histological examination, the interlobular ducts and interstitial connective tissue remained intact. At this time point, the islets of

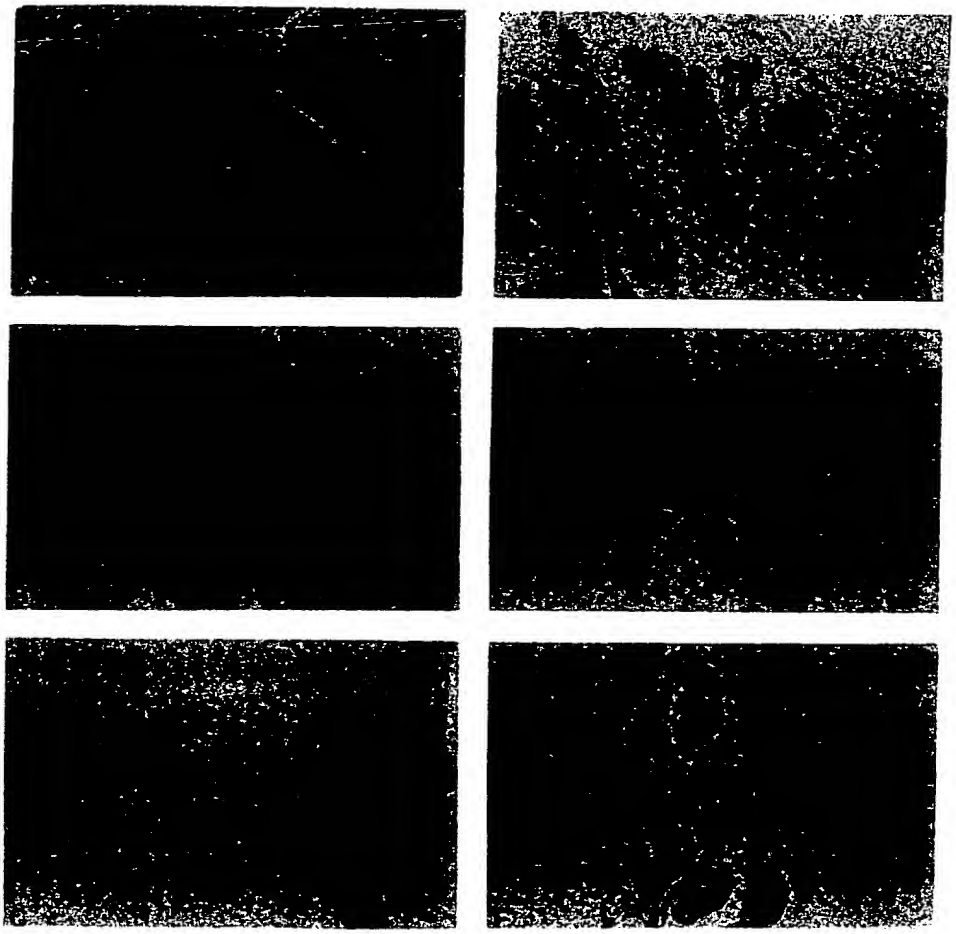

Fig. 2. Histopathology of pancreatic tissue from SJL and B10 mice. SJL and B10 mice were infected with $10^{4.3}$ and $10^{2.7}$ $TCID_{50}$ of CB4-V, respectively. At various times PI, pancreatic tissue was stained with hematoxylin and eosin. (A), PBS-injected control SJL; (B), SJL at 4 days PI; (C), SJL at 8 weeks PI; (D), B10.T(6R) at 4 days PI; (E), B10.S(12R) at 4 days PI; (F) B10.S(12R) at 8 weeks PI. All panels represent equal magnification. Arrows indicate islets of Langerhans.

Langerhans did not show any changes at the light-microscopic level. The degeneration of the exocrine secretory units was progressive so that, by 8 weeks PI, the exocrine pancreas consisted of interlobular ducts and interstitial connective tissue with little or no acinar cells. Still, the islets of Langerhans appeared unchanged and were observed within the remnants of the exocrine pancreas.

Severity and duration of CB4-V-induced hypoglycemia is influenced by the MHC genes

To determine whether susceptibility to CB4-V was influenced by genes within the MHC, five H-2 congenic mouse strains were injected IP with $10^{2.7}$ $TCID_{50}$ of CB4-V, CB4-P or 0.2 ml PBS. Serum glucose concentrations were then monitored at various times PI. A low dosage of virus was chosen since, at this concentration, acinar pancreatitis with concurrent hypoglycemia was induced in SJL mice while the

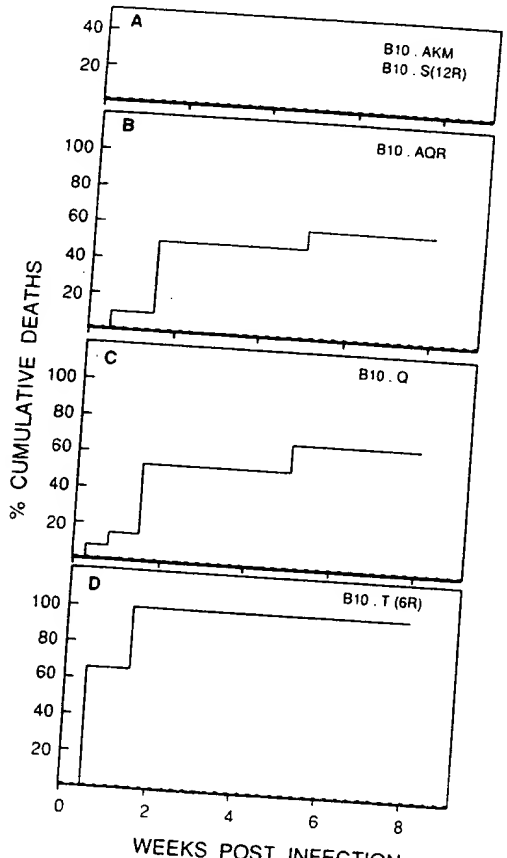

Fig. 3. Cumulative deaths of B10 H-2 congenic mice infected with a low dose, $10^{2.7}$ $TCID_{50}$, of either CB4-V (———) or CB4-P (- - - - -) virus. (A), strains B10.AKM, B10.S(12R); (B), B10.AQR; (C), B10.Q; (D), B10.T(6R).

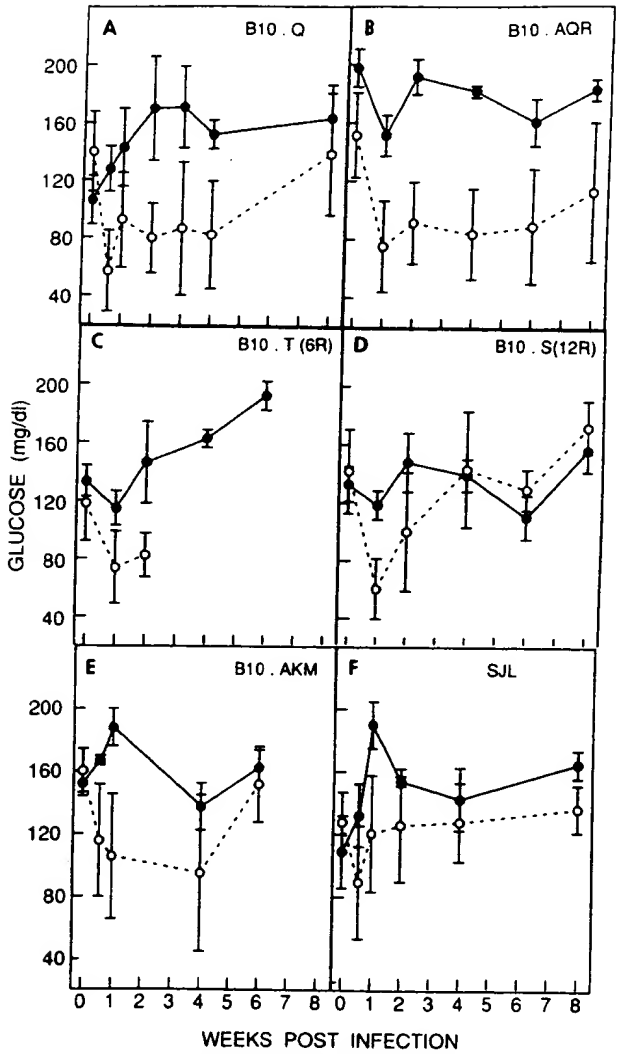

Fig. 4. Serum glucose levels of various mouse strains after IP inoculation with CB4-V (○------○) or with PBS (●———●). Groups of 10–15 4–6-week-old male mice were inoculated with $10^{2.7}$ $TCID_{50}$ of CB4-V and serum glucose concentrations were monitored for 6–8 weeks PI. Infected B10.T(6R) mice were moribund within a 2-week interval. Groups of 5–10 4–6-week-old male mice were inoculated with 0.2 ml of PBS and serum glucose concentrations were monitored for 6–8 weeks. (A), B10.Q; (B), B10.AQR; (C), B10.T(6R); (D), B10.S(12R); (E), B10.AKM; (F), SJL.

mortality rate was 0%. Groups of 5 animals were inoculated with CB4-P or PBS while groups of 10–15 animals were inoculated with CB4-V. These experiments were repeated a total of 3 times and representative results are shown in Figs. 3 and 4. Regardless of strain, mice that were inoculated with either PBS or CB4-P, lived during the follow-up eight weeks PI. In contrast, the B10 H-2 congenic mouse

strains injected with CB4-V fell into either of 2 groups. One group consisting of B10.AQR, B10.Q and B10.T(6R) exhibited 60–100% mortality rates. The second group, consisting of B10.AKM and B10.S(12R), had 0% mortality rates. The severity and duration of hypoglycemia in B10.AKM and B10.S(12R) mice was less than that observed in B10.AQR, B10.Q and B10.T(6R) mice (Fig. 4, Table 1). Mice in the latter group displayed more pronounced shivering, huddling, weight loss and general inactivity than mice in the former group (Table 1). To determine whether non-H-2 genes affected the mortality rate when a low dose of virus was used, CB4-V infected B10.S(12R) mice were compared to CB4-V infected SJL mice which have the same H-2 haplotype i.e. H-2^s. Infected SJL mice displayed symptoms signs to those of infected B10.S(12R) mice and the mortality rate was 0%. Since B10.AQR, B10.Q and B10.T(6R) share the K region of H-2, the severity and duration of hypoglycemia observed in CB4-V-infected B10 H-2 congenic mice was associated with H-$2K^q$.

Of the 5 B10 H-2 congenic mouse strains studied, the most susceptible [B10.T(6R)] and one of the more resistant [B10.S(12R)] strains were chosen for histological examination. Pancreases were harvested from CB4-V-infected 4–6-week-old male mice at different times PI and processed for staining by hematoxylin and eosin (Fig. 2D–F). At 4 days PI, as observed in SJL mice that were infected with a higher titer of virus, in both B10 H-2 congenic mouse strains (Fig. 2D, E), there was degranulation of the acinar cells and loss of the exocrine secretory units. Again, the islets of Langerhans did not show any alterations at the light microscopic level. Unlike the SJL mice, the B10 mice exhibited lymphocytic infiltration into the areas of the exocrine cells. In B10.T(6R) mice, a generalized lymphocytic infiltration that appeared to correlate with destruction of the exocrine pancreas was observed (Fig. 2D). These mice were moribund within 2 weeks PI. For the less susceptible strain, B10.S(12R), focal lymphocytic infiltration was observed (Fig. 2E). This infiltration again correlated with destruction of the exocrine pancreas since tissue free from such infiltrates appeared normal. By 8 weeks PI in the B10.S(12R) mice, foci of lymphocytic infiltration were no longer apparent but regions of degenerative exocrine tissue persisted (Fig. 2F). Unlike CB4-V-infected mice, pancreases harvested from B10.T(6R) and B10.S(12R) congenic mice infected with $10^{2.7}$ $TCID_{50}$ of the prototypical virus (CB4-P) showed only minor changes in the exocrine tissue, such as shrunken acinar cells with smaller nuclei; lymphocytic infiltrates were not observed (data not shown).

Isolation of virus from organs of infected mice

To determine whether CB4 virus replicated differentially in B10.T(6R) and B10.S(12R) mice, various organs were harvested from mice infected with $10^{2.7}$ $TCID_{50}$ of virus at 4 days PI when viral titers were maximal (data not shown). In each experiment, organs from 3–4 mice were pooled and homogenates were titered for the presence of infectious virus (Table 2). Both CB4-V and CB4-P replicated well in B10.T(6R) and B10.S(12R) mice. Viral titers were higher in the pancreas and spleen than in the kidney or heart. In both mouse strains, CB4-V replicated

TABLE 2

Virus titers obtained from various organs of infected B10 H-2 congenic mice

Virus	Mouse strain	Virus titer [a] ($-\log_{10}$ $TCID_{50}$/g tissue)			
		Heart	Kidney	Spleen	Pancreas
CB4-P	B10.T(6R)	3.2	3.9	7.2	6.9
	B10.S(12R)	2.8	4.0	5.4	7.1
CB4-V	B10.T(6R)	3.0	ND [b]	4.7	6.0
	B10.S(12R)	3.7	3.5	3.9	5.2

[a] Virus titers from 3 independent experiments were determined and were reproducible within a 10-fold range. The microtiter $TCID_{50}$ assay used in these experiments is accurate to $10^{0.5}$ (Minor, 1985). Representative results of a single experiment are shown in this Table.

[b] Not detected.

maximally in pancreas, which probably reflects the fact that this virus was derived by passaging pancreatic homogenates in vivo.

Discussion

The pathogenesis of Coxsackie B virus infections is complex and has been shown to be influenced by both host factors such as age, sex and strain (Khatib et al., 1980) and by intrinsic factors such as virus type and passage history (Toniolo et al., 1982). These experiments addressed whether or not infection by CB4 viruses (CB4-P and CB4-V) is dependent on MHC haplotype.

A pancreatropic variant, CB4-V, was generated by passaging virus in mice via pancreatic homogenates. In SJL mice, this virus caused a prolonged and severe hypoglycemia with concurrent pancreatis as is shown by degeneration of the acinar cells of the exocrine pancreas. Lymphocytic infiltration of pancreatic tissue was not observed. Infection of various B10 H-2 congenic mouse strains revealed that the development of hypoglycemia with accompanying pancreatitis was MHC-independent. However, disease outcome, as monitored by the severity and duration of hypoglycemia and by mortality rate, was associated with the H-2 haplotype. Three strains of mice, B10.AQR, B10.Q and B10.T(6R) had high mortality rates (43–100%) while mice of strains B10.AKM and B10.S(12R) were not killed by the virus. Thus, in B10 mice, the severity of disease induced by CB4-V correlated with the $H\text{-}2K^q$ locus. Although CB4-P replicated well in both B10.T(6R) and B10.S(12R), these mice were normoglycemic and did not appear ill during the follow-up eight weeks post-infection. This suggested that like CB4-V, infection of B10 H-2 congenic mice with CB4-P was MHC-independent.

Since the K locus gene product can act to restrict the response of cytotoxic T lymphocytes (CTL) to viral antigens, several possibilities can be proposed to explain the correlation of severity of CB4-V infection with K^q. One possibility is that K^q acts as a major restriction element for the antigens of CB4-V, resulting in an increased CTL response in the B10.AQR, B10.Q and B10.T(6R) mouse strains as

compared to the B10.AKM and B10.S(12R) strains. Thus, pancreatic damage would be due primarily to immune-mediated destruction of infected acinar cells by CTL. As an example, susceptibility to lymphocytic choriomeningitis virus (LCMV) is linked to H-2D^q (Zinkernagel et al., 1985). LCMV can persist in pancreatic β cells of adult mice and is associated with aberrations in blood glucose concentrations (Tishon and Oldstone, 1987). Furthermore, LCMV-induced disease is the result of a T cell-mediated pathophysiological mechanism (Zinkernagel et al., 1985). A second possibility is that K^q does not function as a restriction element for CB4-V. This lack of recognition by CTL of viral-infected cells should result in higher titers of virus in infected tissue due to increased viral replication and spread. Pancreatic damage in this case would be due primarily to viral-mediated destruction. Khatib et al. (1987) have suggested that myocardial damage in the acute phase of CB3 infection is caused by direct virus cytopathogenicity rather than by host immune response. We examined whether CB4 virus replicated differentially in a susceptible [B10.T(6R)] and a resistant [B10.S(12R)] strain of mice (Table 2). Both CB4-V and CB4-P replicated to high titers in the pancreas of both strains of mice. However, CB4-V induced a severe hypoglycemia in B10.T(6R) mice, which succumbed to viral infection. CB4-V-infected B10.S(12R) mice exhibited a much milder hypoglycemia and did not succumb to viral infection. Since preferential replication of CB4-V in the most susceptible mouse strain, B10.T(6R), was not observed, this argues against K^q influencing viral replication and destruction. An extension of this argument would be that direct viral destruction is not the major mechanism of pancreatic injury. This is also supported by the fact that CB4-P replicated to high titers in the pancreas of both B10.T(6R) and B10.S(12R) mice yet histological examination revealed no destruction of pancreatic tissue. Histological examination of pancreatic tissue from CB4-V infected B10 H-2 congenic mice revealed an association between acute destruction of the exocrine pancreas and lymphocytic infiltration. In the resistant B10.S(12R) mice, focal infiltrates were observed in the exocrine tissue. However, a generalized infiltration was observed in the exocrine tissue of the susceptible B10.T(6R) mice. This generalized lymphocytic infiltration may correlate with immune-mediated destruction of pancreatic tissue in B10 mice but not in SJL mice. To determine whether pancreatic damage in CB4-V infected mice is a result of immune-mediated destruction, we are currently testing whether K^q acts as a restriction element for viral antigens by measuring the CTL response of the B10 H-2 congenic mice to CB4-V. In addition to examining the role of host genes in the pathogenesis of CB4 virus infections, hybrid viruses have been constructed from cDNA clones of both CB4-V and CB4-P to localize the gene(s) involved in disease induction (Ramsingh et al., manuscript in preparation).

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References

Dalldorf, G. and Gifford, R. (1952) Adaptation of group B coxsackie virus to adult mouse pancreas. J. Exp. Med. 96, 491–497.

Gamble, D.R., Kinsley, M.L., Fitzgerald, M.G., Bolton, R. and Taylor, K.W. (1969) Viral antibodies in diabetes mellitus. Br. Med. J. 3, 627–630.

Khatib, R., Chason, J.L., Silberberg, B.K. and Lerner, A.M. (1980) Age-dependent pathogenicity of group B coxsackieviruses in Swiss-Webster mice: infectivity for myocardium and pancreas. J. Infect. Dis. 141, 394–403.

Khatib, R., Probert, A., Reyes, M.P., Khatib, G. and Chason, J.L. (1987) Mouse strain-related variation as a factor in the pathogenesis of Coxsackie B3 murine myocarditis. J. Gen. Virol. 68, 2981–2988.

Lansdown, A.B.G. (1976) Pathological changes in the pancreas of mice following infection with Coxsackie B viruses. Br. J. Exp. Pathol. 57, 331–338.

Minor, P.D. (1985) Growth, assay and purification of picornaviruses. In: B.W.J. Mahy (Ed.), Virology, a Practical Approach, pp. 25–41. IRL Press, Oxford, U.K.

Onodera, T., Yoon, Y.W., Brown, K.S. and Notkins, A.L. (1978) Evidence for a single locus controlling susceptibility to virus-induced diabetes mellitus. Nature (London) 274, 693–696.

Pappenheimer, A.M., Kunz, L.J. and Richardson, S. (1951) Passage of Coxsackie virus (Connecticut-5 strain) in adult mice with production of pancreatic disease. J. Exp. Med. 94, 45–64.

Raabo, E. and Terkildsen, T.C. (1960) On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12, 402–407.

Ross, M.E., Hayashi, K. and Notkins, A.L. (1974) Virus-induced pancreatic disease: alterations in concentration of glucose and amylase in blood. J. Infect. Dis. 129, 669–676.

Ross, M.E., Onodera, T., Brown, K. and Notkins, A.L. (1976) Virus-induced diabetes mellitus. IV. Genetic and environmental factors influencing the development of diabetes after infection with the M variant of encephalomyocarditis virus. Diabetes 25, 190–197.

Tishon, A. and Oldstone, M.B.A. (1987) Persistent virus infection associated with chemical manifestations of diabetes. II. Role of viral strain, environmental insult, and host genetics. Am. J. Pathol. 126, 61–72.

Toniolo, A., Onodera, T., Jordan, G., Yoon, J.W. and Notkins, A.L. (1982) Virus-induced diabetes mellitus. Glucose abnormalities produced in mice by the six members of the Coxsackie B virus group. Diabetes, 31, 496–499.

Yoon, J.W., Austin, M., Onodera, T. and Notkins, A.L. (1979) Virus-induced diabetes mellitus. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N. Engl. J. Med. 300, 1173–1179.

Yoon, J.W. and Notkins, A.L. (1976) Virus-induced diabetes mellitus. VI. Genetically determined host differences in the replication of encephalomyocarditis virus in pancreatic beta cells. J. Exp. Med. 143, 1170–1185.

Yoon, J.W., Onodera, T., Jenson, A.B. and Notkins, A.L. (1978a) Virus-induced diabetes mellitus. XI. Replication of Coxsackie virus B3 in human pancreatic beta cell cultures. Diabetes 27, 778–781.

Yoon, J.W., Onodera, T. and Notkins, A.L. (1978b) Virus-induced diabetes mellitus. XV. Beta cell damage and insulin-dependent hyperglycemia in mice infected with Coxsackie B4. J. Exp. Med. 148, 1068–1080.

Zinkernagel, R.M., Pfau, C.J., Hengartner, H. and Althage, A. (1985) Susceptibility to murine lymphocytic choriomeningitis maps to class 1 MHC genes: a model for MHC/disease associations. Nature (London) 316, 814–817.

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1: S39291. Coxsackievirus B4...[gi:250908]

LOCUS S39291 3298 bp RNA linear VRL 03-AUG-1999
DEFINITION Coxsackievirus B4 VP4 gene, partial sequence; VP2 (VP2) gene, partial cds; VP3 gene, partial sequence; and VP1 (VP1) gene, partial cds.
ACCESSION S39291
VERSION S39291.1 GI:250908
KEYWORDS .
SOURCE Human coxsackievirus B4
ORGANISM Human coxsackievirus B4
Viruses; ssRNA positive-strand viruses, no DNA stage; Picornaviridae; Enterovirus.
REFERENCE 1 (bases 1 to 3298)
AUTHORS Ramsingh,A., Araki,H., Bryant,S. and Hixson,A.
TITLE Identification of candidate sequences that determine virulence in Coxsackievirus B4
JOURNAL Virus Res. 23 (3), 281-292 (1992)
PUBMED 1320798
REMARK GenBank staff at the National Library of Medicine created this entry [NCBI gibbsq 108105] from the original journal article.

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